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Sibinga et al.

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(54) **INTRACELLULAR DOMAIN OF A MAMMALIAN FAT1 (FAT1IC)**

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(51) **Int. Cl.**
A61K 38/16 (2006.01)
C07K 14/00 (2006.01)

(52) **U.S. Cl.**
USPC **514/12**; 530/350

(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

Provided is an extracellular domain of a mammalian Fat1 ($Fat1_{EC}$). Also provided is an intracellular domain of a mammalian Fat1 ($Fat1_{IC}$). Additionally provided is a vector comprising a nucleic acid sequence encoding the $Fat1_{EC}$. A vector comprising a nucleic acid sequence encoding the $Fat1_{IC}$ is further provided. Also, a vascular stent coated with the $Fat1_{EC}$ is provided. Further provided is a vascular stent coated with a Fat1 ligand that activates Fat1. A method of treating a patient at risk for restenosis of a blood vessel is additionally provided. Further, methods of treating an injured blood vessel in a patient is provided. A method of treating a patient at risk for restenosis of a blood vessel or having an injured blood vessel is also provided.

5 Claims, 10 Drawing Sheets

FIG. 1

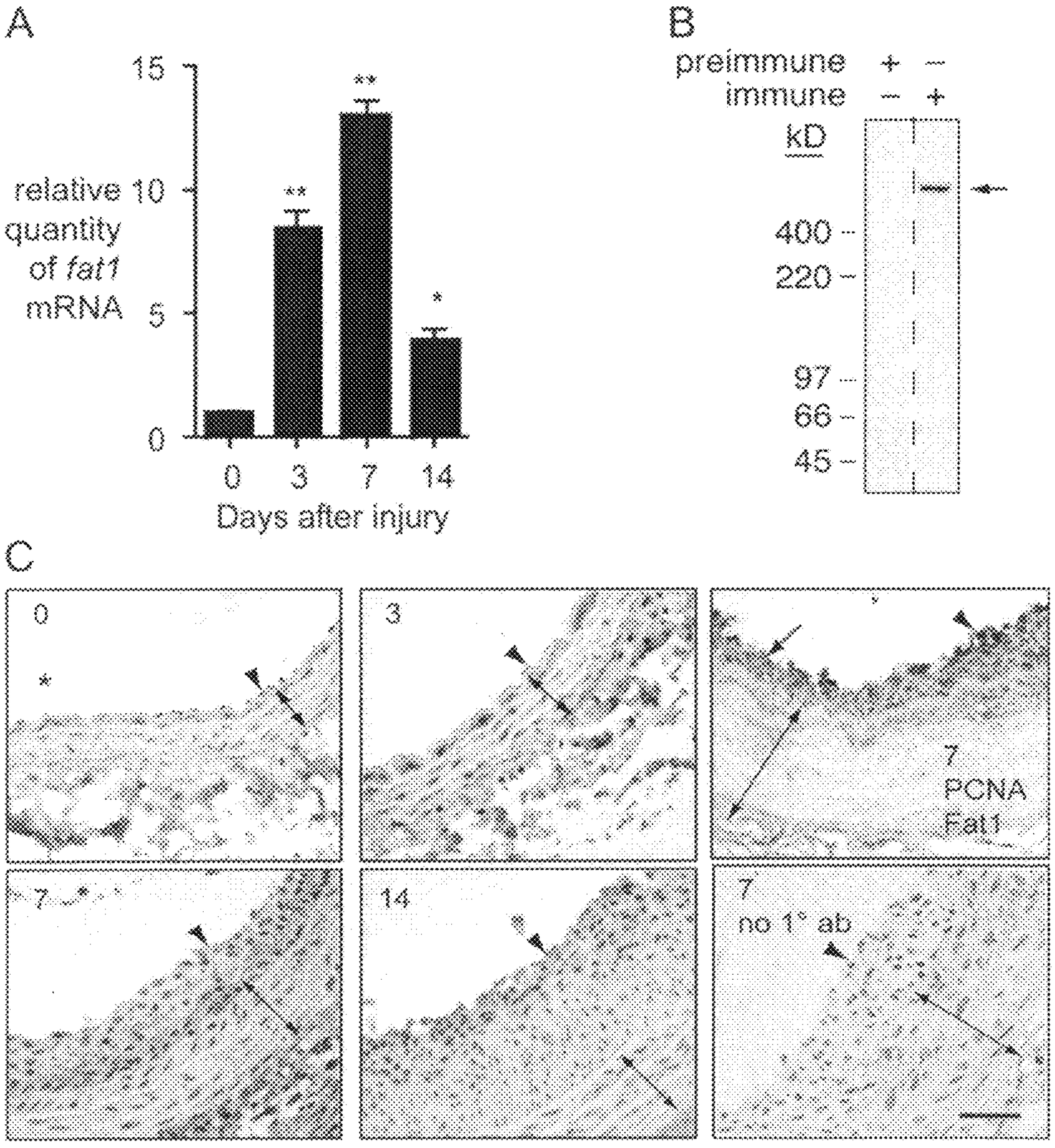

A
relative quantity of fat1 mRNA
15
10
5
0
**
**
**
*
0
3
7
14
Days after injury
B
preimmune + −
immune − +
kD
400
220
97
66
45
C
0
*
3
7
PCNA
Fat1
7
14
7
no 1° ab

FIG. 2

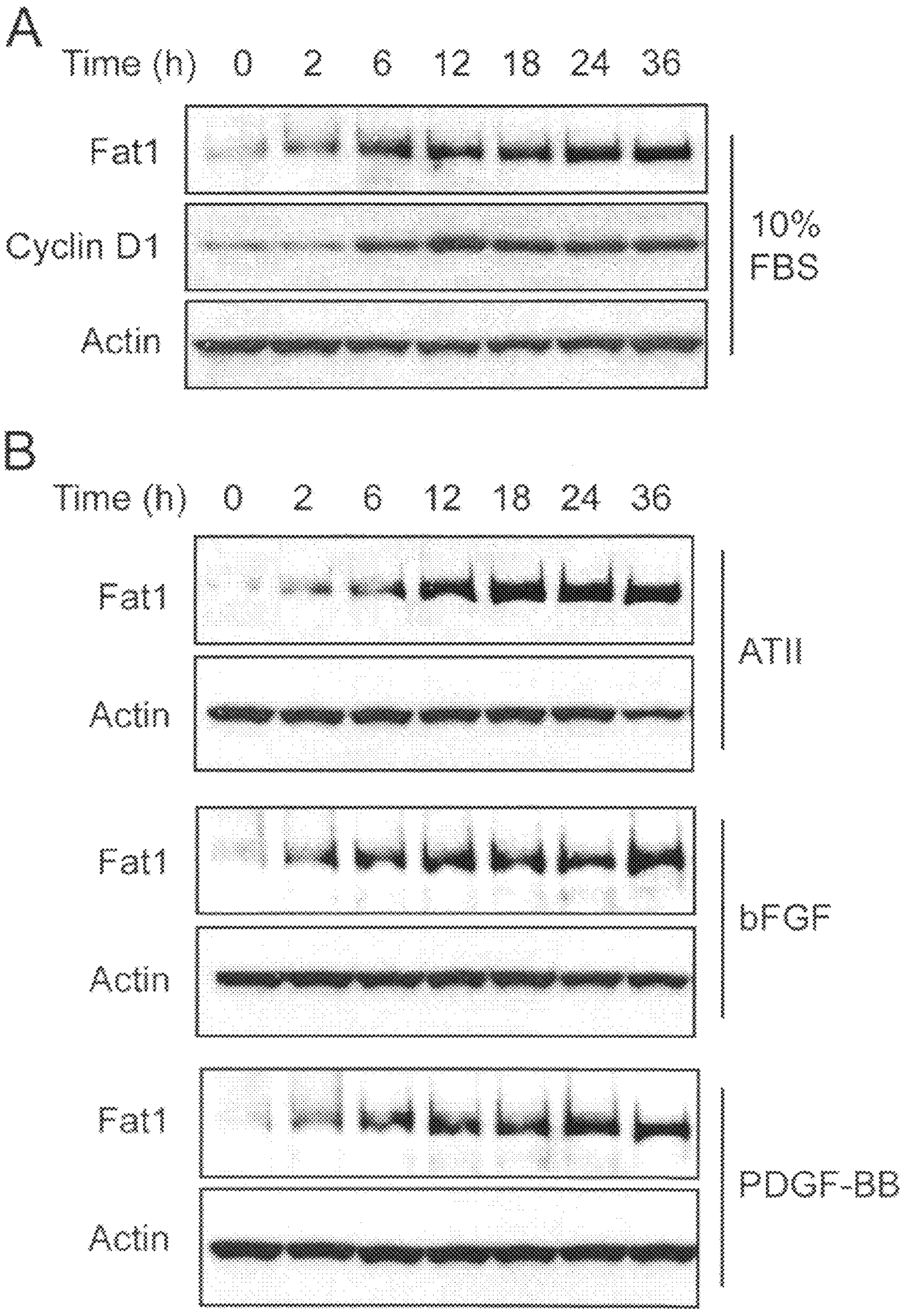

A
Time (h) 0 2 6 12 18 24 36
Fat1
Cyclin D1
Actin
10% FBS
B
Time (h) 0 2 6 12 18 24 36
Fat1
Actin
ATII
Fat1
Actin
bFGF
Fat1
Actin
PDGF-BB

FIG. 3

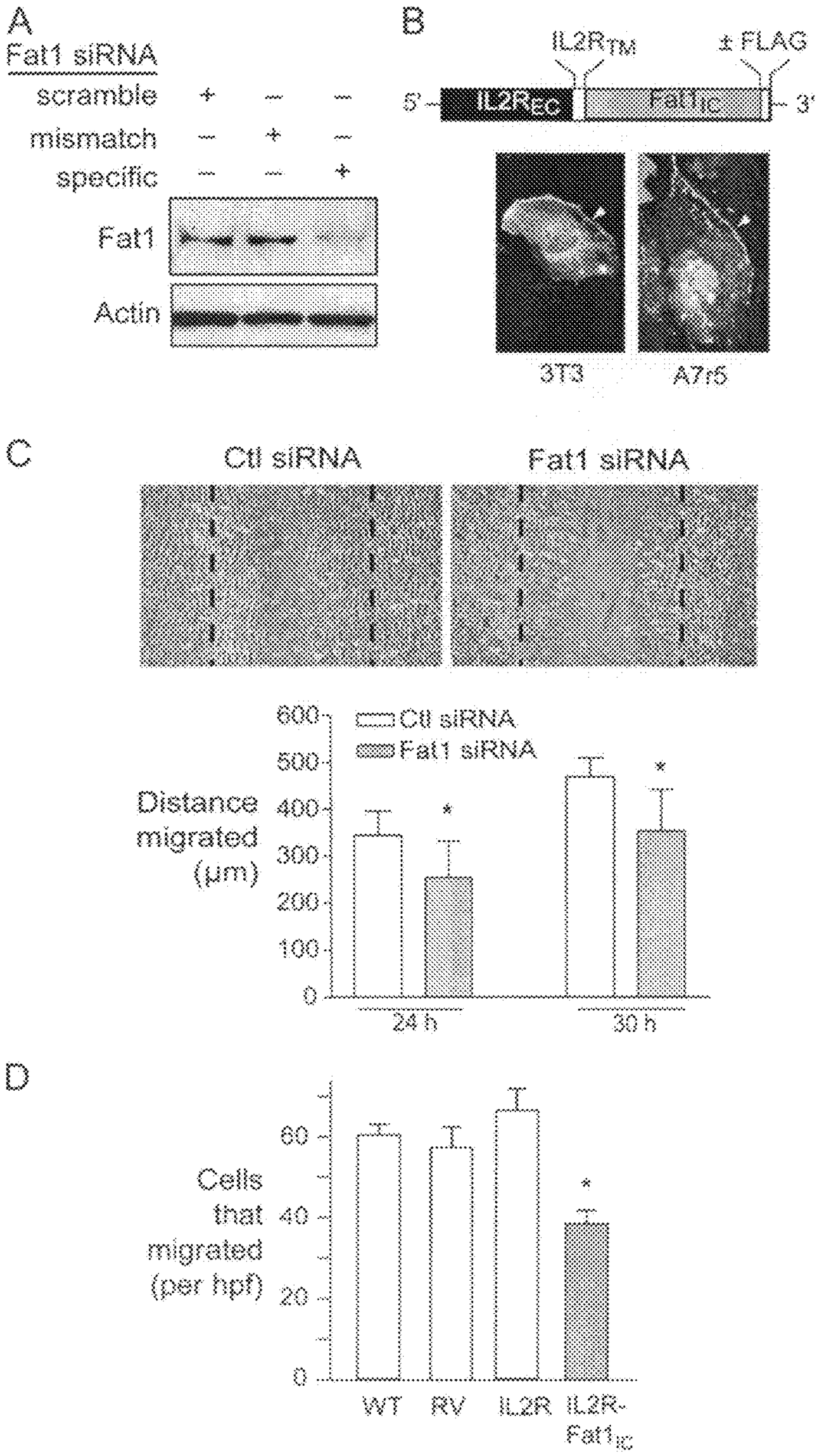
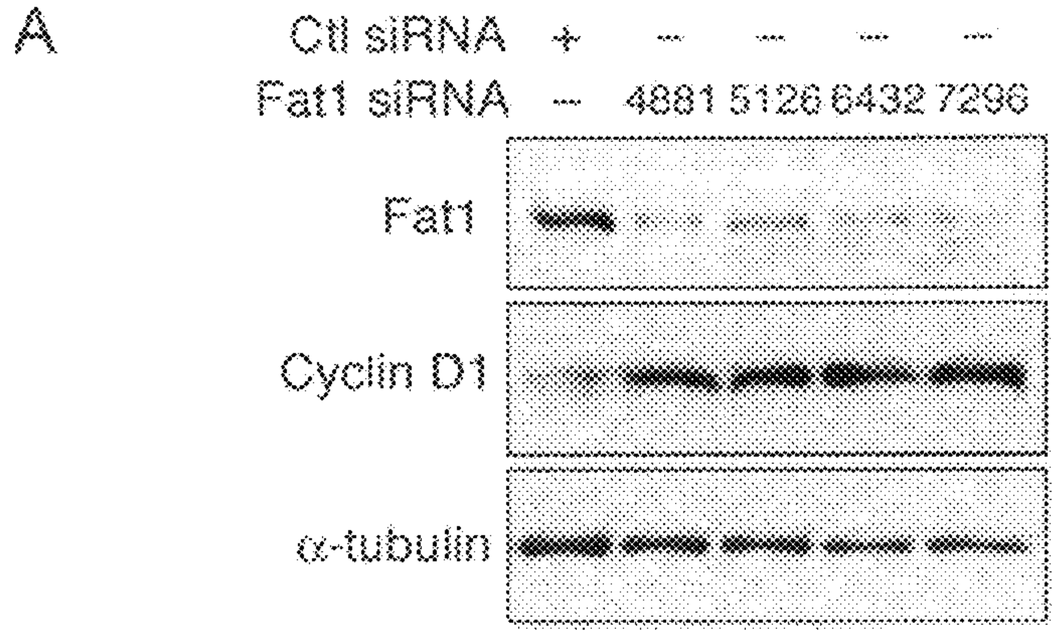
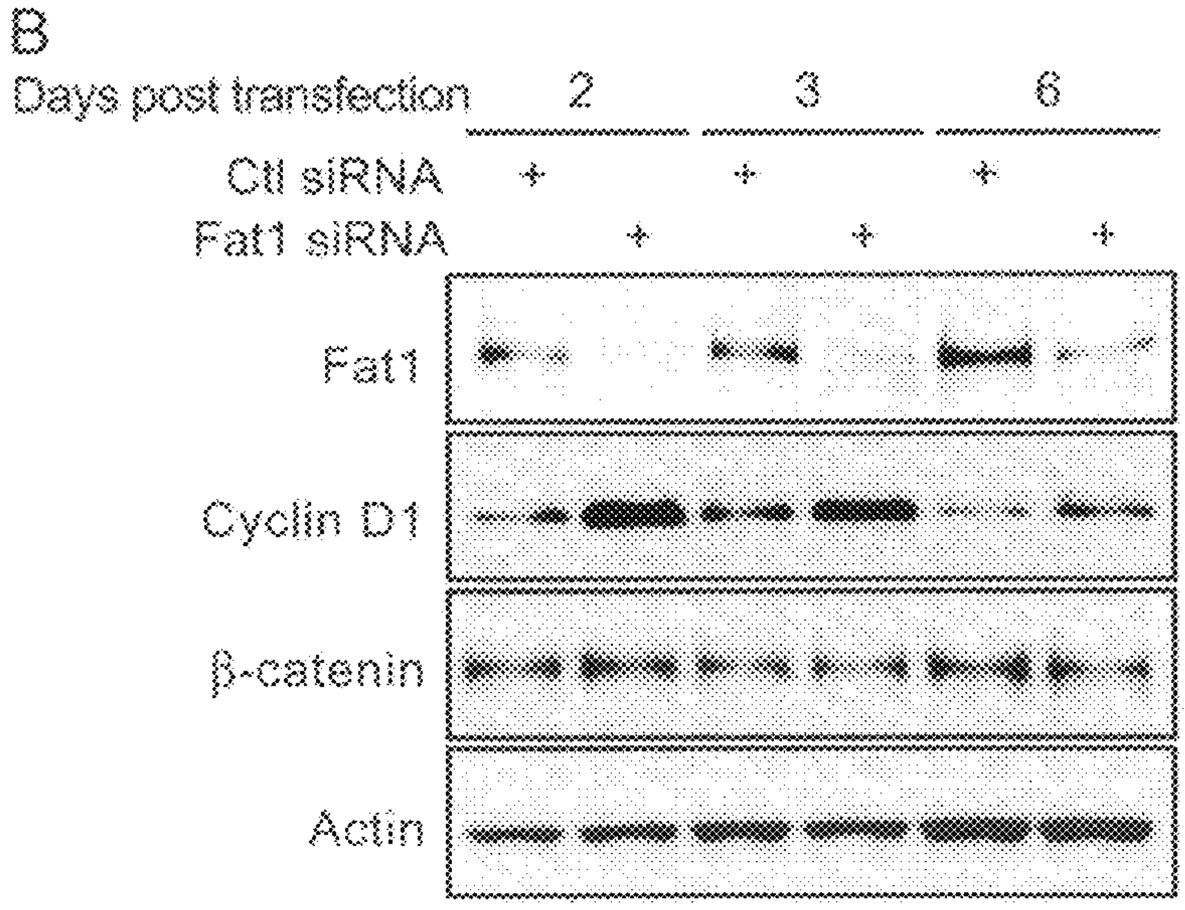

A
Fat1 siRNA
scramble + – –
mismatch – + –
specific – – +
Fat1
Actin
B
IL2R$_{TM}$
± FLAG
5'
IL2R$_{EC}$
Fat1$_{IC}$
3'
3T3
A7r5
C
Ctl siRNA
Fat1 siRNA
Distance migrated (μm)
600
500
400
300
200
100
0
Ctl siRNA
Fat1 siRNA
*
*
24 h
30 h
D
Cells that migrated (per hpf)
60
40
20
0
*
WT
RV
IL2R
IL2R-Fat1$_{IC}$

FIG. 4


A
Ctl siRNA + – – – –
Fat1 siRNA – 4881 5126 6432 7296
Fat1
Cyclin D1
α-tubulin


B
Days post transfection 2 3 6
Ctl siRNA + + +
Fat1 siRNA + + +
Fat1
Cyclin D1
β-catenin
Actin

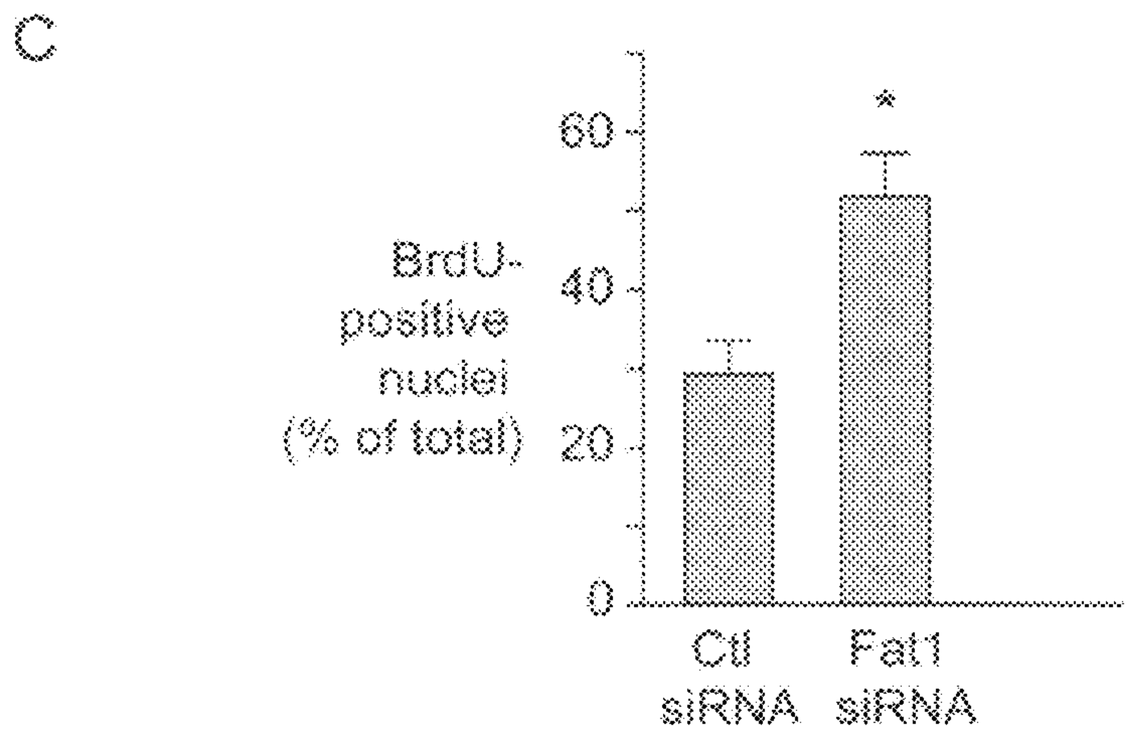

C
BrdU-positive nuclei (% of total)
60
40
20
0
*
Ctl siRNA
Fat1 siRNA

FIG. 5

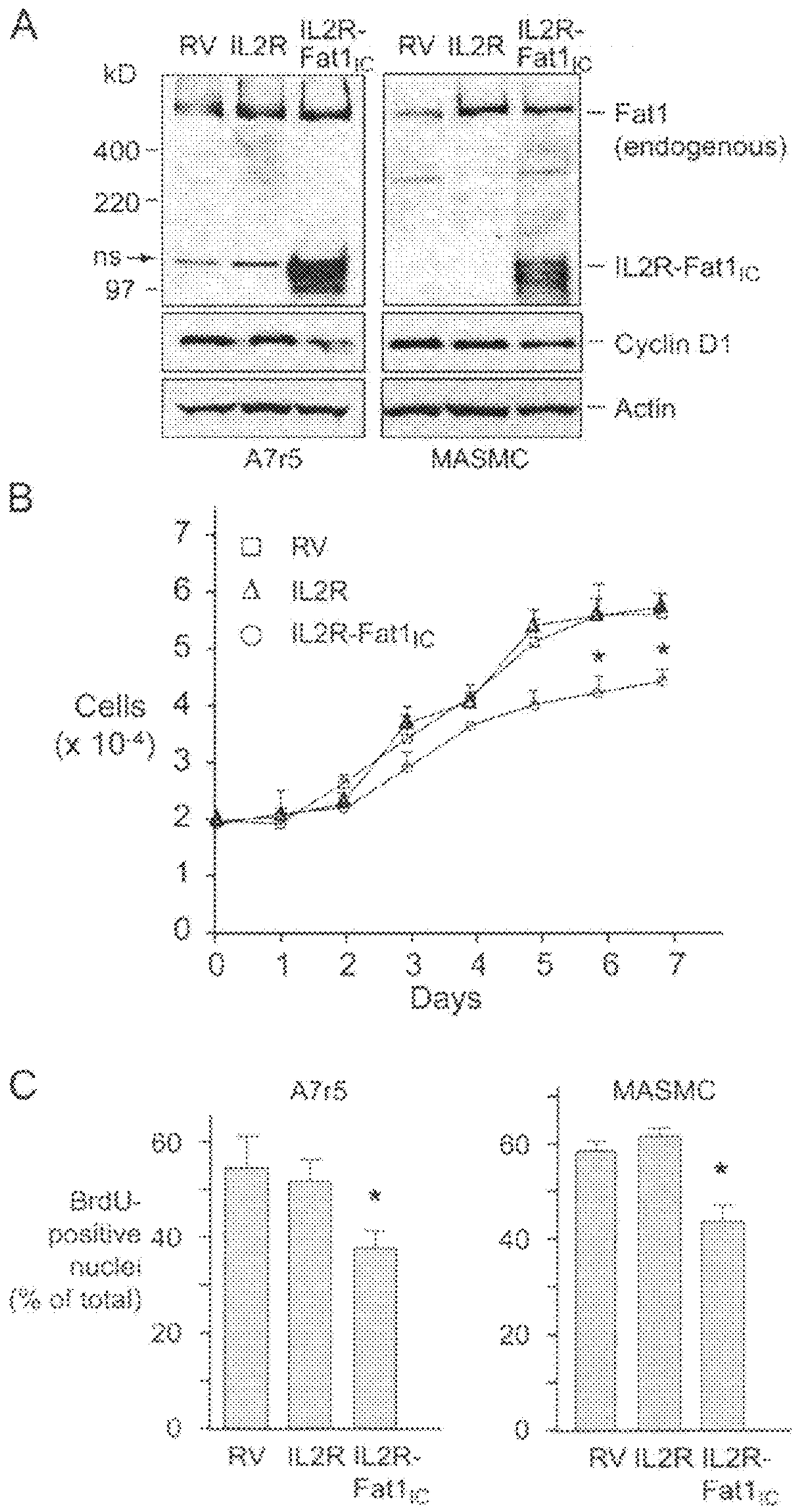

A
RV
IL2R
IL2R-Fat1IC
kD
400
220
ns
97
Fat1 (endogenous)
IL2R-Fat1IC
Cyclin D1
Actin
A7r5
MASMC
B
RV
IL2R
IL2R-Fat1IC
Cells (x 10⁴)
Days
C
A7r5
MASMC
BrdU-positive nuclei (% of total)

FIG. 6

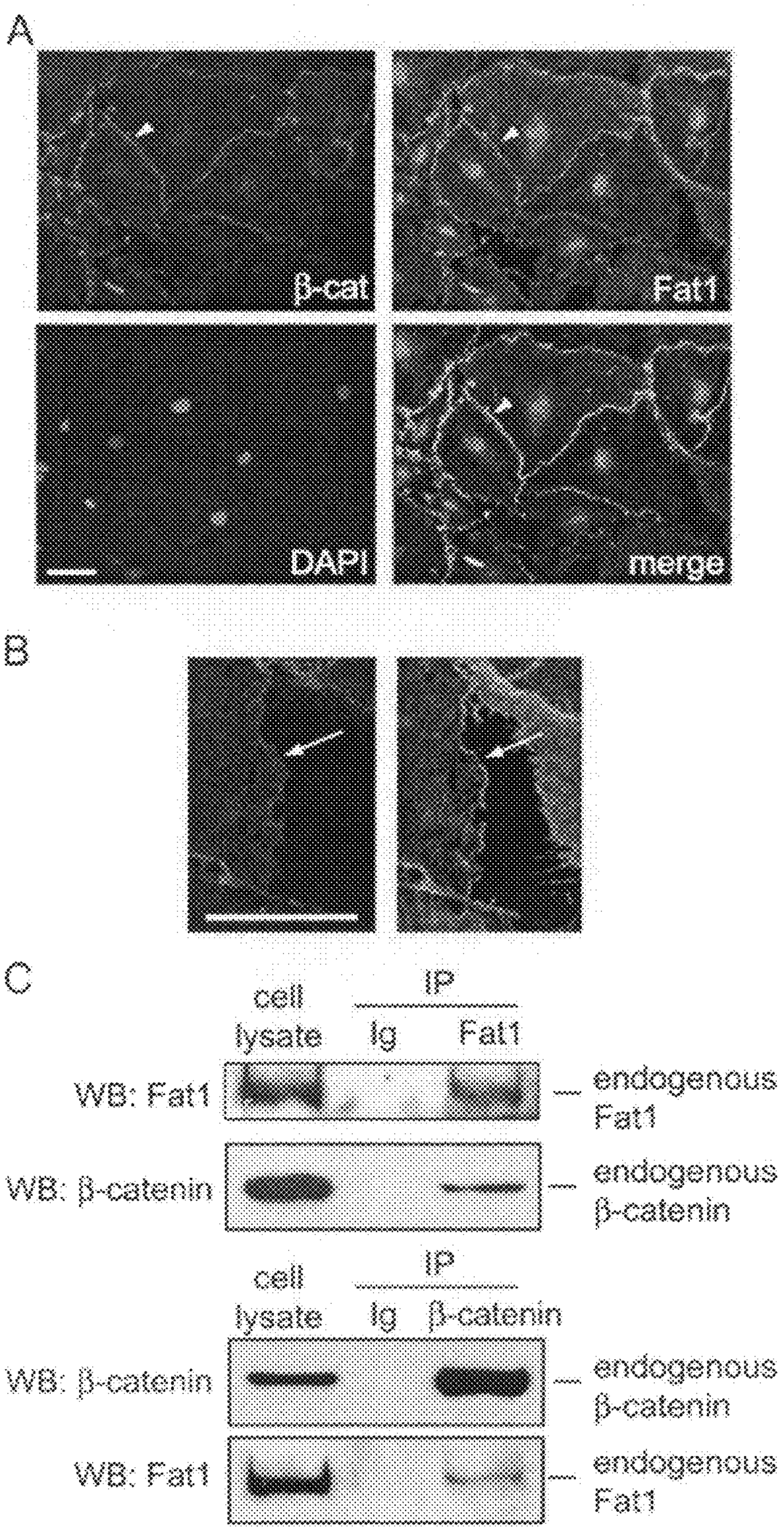

A
β-cat
Fat1
DAPI
merge
B
C
cell lysate
IP
Ig
Fat1
WB: Fat1
endogenous Fat1
WB: β-catenin
endogenous β-catenin
cell lysate
IP
Ig
β-catenin
WB: β-catenin
endogenous β-catenin
WB: Fat1
endogenous Fat1

FIG. 7

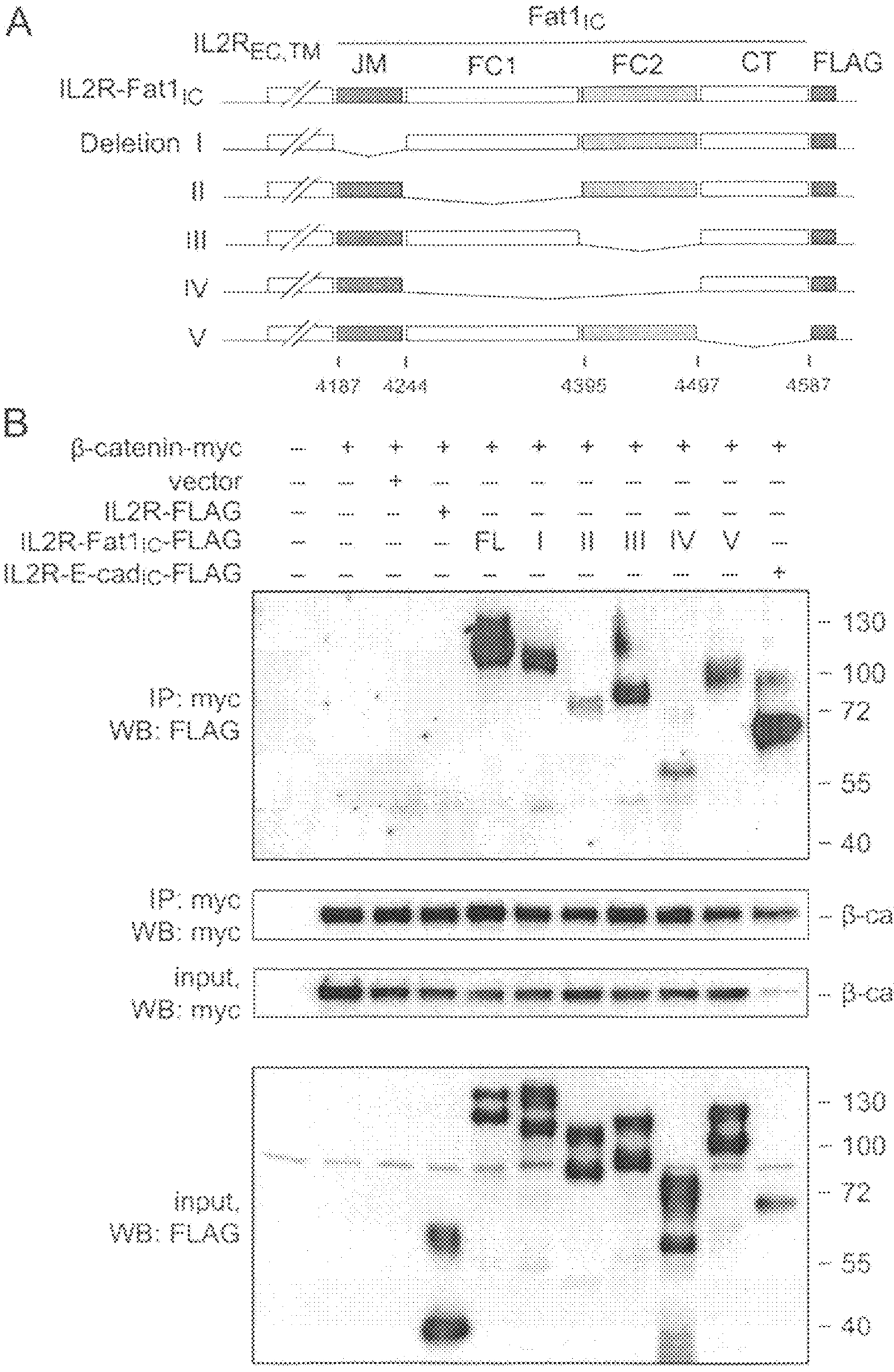

A
Fat1IC
IL2REC.TM
JM
FC1
FC2
CT
FLAG
IL2R-Fat1IC
Deletion I
II
III
IV
V
4187
4244
4395
4497
4587
B
β-catenin-myc
vector
IL2R-FLAG
IL2R-Fat1IC-FLAG
IL2R-E-cadIC-FLAG
FL
I
II
III
IV
V
IP: myc
WB: FLAG
130 kD
100
72
55
40
IP: myc
WB: myc
β-catenin
input,
WB: myc
β-catenin
input,
WB: FLAG
130
100
72
55
40

FIG. 8

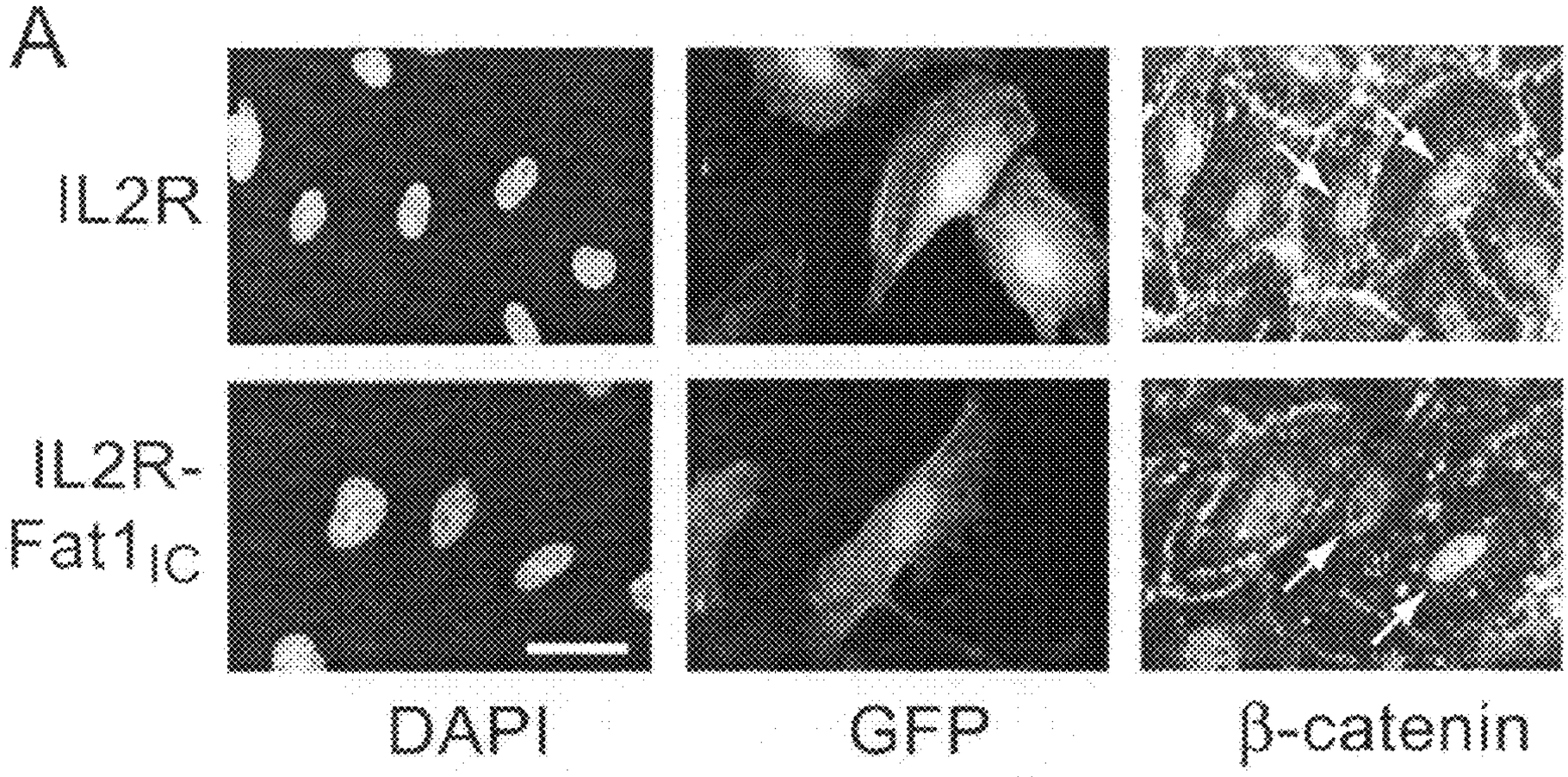

A
IL2R
IL2R-Fat1IC
DAPI
GFP
β-catenin
B
IL2R
IL2R-Fat1IC
β-catenin
Lamin A/C
M C N M C N

FIG. 9

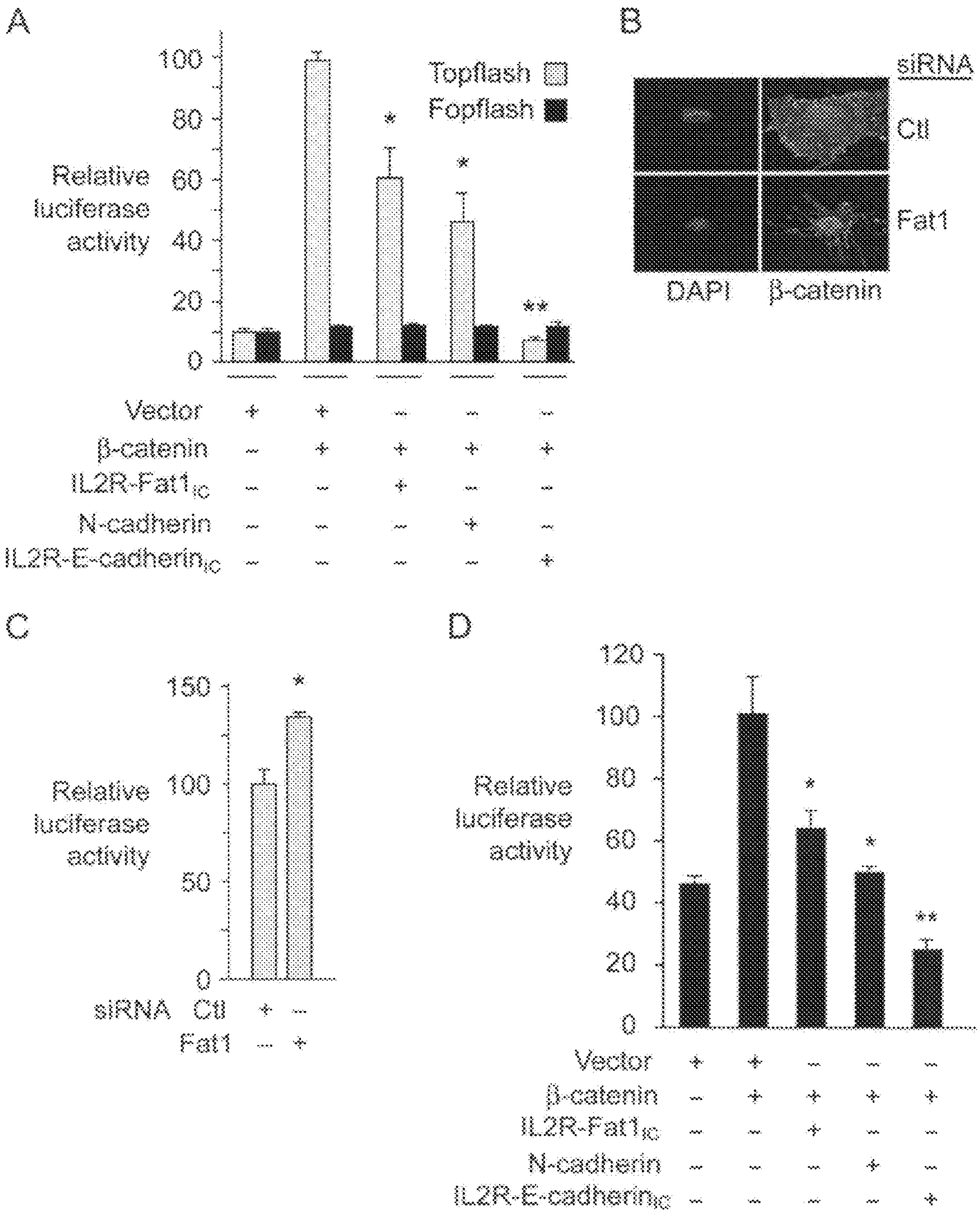
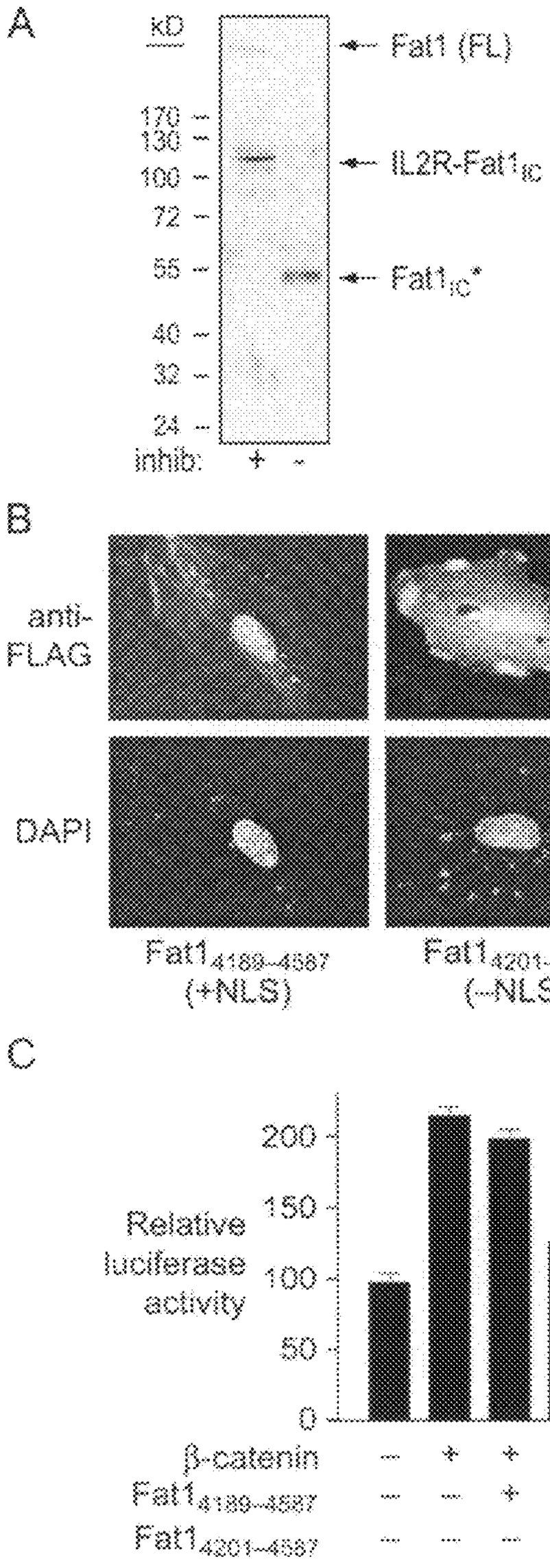

A
Relative luciferase activity
100
80
60
40
20
0
Topflash
Fopflash
*
*
**
Vector + + – – –
β-catenin – + + + +
IL2R-Fat1IC – – + – –
N-cadherin – – – + –
IL2R-E-cadherinIC – – – – +
B
siRNA
Ctl
Fat1
DAPI
β-catenin
C
Relative luciferase activity
150
100
50
0
*
siRNA Ctl + –
Fat1 – +
D
Relative luciferase activity
120
100
80
60
40
20
0
*
*
**
Vector + + – – –
β-catenin – + + + +
IL2R-Fat1IC – – + – –
N-cadherin – – – + –
IL2R-E-cadherinIC – – – – +

FIG. 10


A
kD
170
130
100
72
55
40
32
24
inhib:
+
-
Fat1 (FL)
IL2R-Fat1IC
Fat1IC*
B
anti-FLAG
DAPI
Fat14189–4587 (+NLS)
Fat14201–4587 (–NLS)
C
Relative luciferase activity
200
150
100
50
0
*
β-catenin
Fat14189–4587
Fat14201–4587

INTRACELLULAR DOMAIN OF A MAMMALIAN FAT1 (FAT1IC)

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/928,216, filed May 8, 2007, the contents of which are hereby incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The invention was made with government support under grant number R01 HL67944-01 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention generally relates to compositions and treatments for vascular disorders. More specifically, the invention provides compositions comprising Fat1 or isolated domains thereof, and methods of using these and other compositions with methods of vascular repair such as angioplasty and coronary bypass surgery.

(2) Description of the Related Art

Vascular remodeling is a critical part of the pathogenesis of clinically important vascular disorders such as atherosclerosis, restenosis after angioplasty, and saphenous vein graft disease (Shanahan and Weissberg, 1998; Owens et al., 2004). Despite considerable study, the molecular mechanisms that control vascular smooth muscle cell (VSMC) activities during vascular remodeling are not fully understood. Recent reports linking cadherins to VSMC regulation (Jones et al., 2002; Slater et al., 2004; Uglow et al., 2003) suggest that these transmembrane adhesion proteins, characterized extensively as major mediators of epithelial cell homeostasis, may also be important in vascular remodeling.

Cadherins are involved in Ca^{2+}-dependent cell-cell adhesion, intracellular junction assembly, and tissue morphogenesis during development (Yap et al., 1997; Angst et al., 2001; Wheelock and Johnson, 2003b). Major subdivisions of the large cadherin superfamily include the classical cadherins and the protocadherins (Gallin, 1998; Yagi and Takeichi, 2000; Angst et al., 2001). The extracellular domains of these proteins share a unique structure, the cadherin motif, which is repeated in tandem in variable numbers. Classical cadherins function as homophilic adhesive molecules, and both extracellular and cytoplasmic domains contribute to this function. Classical cadherin cytoplasmic domains interact with β-catenin and plakoglobin (Huber and Weis, 2001; Takeichi, 1995), members of the armadillo gene family of transcription factors. This interaction effectively sequesters β-catenin away from the nucleus, limits its transcriptional activity (Sadot et al., 1998; Kaplan et al., 2001; Simcha et al., 2001), and thus links cadherins to the canonical Wnt signaling pathway, a major determinant of cellular activity during development (Bhanot et al., 1999; Jamora et al., 2003; Nelson and Nusse, 2004).

Like classical cadherins, protocadherins have extracellular domains capable of Ca^{2+}-dependent, homophilic interaction (Suzuki, 2000). Protocadherin cytoplasmic domains, on the other hand, are structurally divergent from those of the classical cadherins, and less is known about their function.

Sequestration and inhibition of β-catenin by protocadherins has not been described.

Although mammalian Fat1 genes (Dunne et al., 1995; Ponassi et al., 1999; Cox et al., 2000) were initially characterized as homologues of the *Drosophila* protein Fat (Mahoney et al., 1991), recent bioinformatics analysis indicates that Fat1 is more closely related to *Drosophila* Fat-like (Ftl) (Castillejo-Lopez et al., 2004). In *Drosophila*, Ftl is expressed apically in luminal tissues such as trachea, salivary glands, proventriculus, and hindgut (Castillejo-Lopez et al., 2004). Silencing of ftl results in the collapse of tracheal epithelia, and it has been suggested that Ftl is required for morphogenesis and maintenance of tubular structures of ectodermal origin.

Like *Drosophila* Fat and Ftl, mammalian Fat1 is remarkable for its very large size (~4600 aa). It has a huge extracellular domain that contains 34 cadherin repeats, 5 EGF-like repeats, and 1 laminin A-G motif, a single transmembrane region, and a cytoplasmic tail of ~400 aa (Dunne et al., 1995). Sequences within the Fat1 cytoplasmic domain ($Fat1_{IC}$) show limited similarity to β-catenin binding regions of classical cadherins (Dunne et al., 1995).

SUMMARY OF THE INVENTION

The inventors have discovered that Fat1 regulates growth and migration of vascular smooth muscle cells (VSMCs).

The invention is directed to an extracellular domain of a mammalian Fat1 ($Fat1_{EC}$), the extracellular domain comprising amino acids equivalent to amino acids 22-4174 of a wild-type mouse Fat1 having the amino acid sequence of SEQ ID NO:1, where the extracellular domain does not comprise the entire mammalian Fat1.

The invention is also directed to an intracellular domain of a mammalian Fat1 ($Fat1_{IC}$), the intracellular domain comprising amino acids equivalent to amino acids 4199-4598 of a wild-type mouse Fat1 having the amino acid sequence of SEQ ID NO:1, where the extracellular domain does not comprise the entire mammalian Fat1.

The invention is additionally directed to a vector comprising a nucleic acid sequence encoding the above-described $Fat1_{EC}$, wherein the vector is capable of expressing the $Fat1_{EC}$ in a mammalian cell.

Additionally, the invention is directed to a vector comprising a nucleic acid sequence encoding the above-described $Fat1_{IC}$, wherein the vector is capable of expressing the $Fat1_{IC}$ in a mammalian cell.

The invention is further directed to a vascular stent coated with the above-described $Fat1_{EC}$.

Also, the invention is directed to vascular stents coated with a Fat1 ligand that activates Fat1.

Further, the invention is directed to a method of treating a patient at risk for restenosis of a blood vessel, the method comprising inserting the above-described vascular stent into the blood vessel at the site of the risk.

The invention is also directed to a method of treating an injured blood vessel in a patient. The method comprises administering the above-described $Fat1_{IC}$ or the above-described $Fat1_{IC}$ vector to the injured blood vessel in a manner sufficient to treat the injured blood vessel.

The invention is additionally directed to a method of treating a patient at risk for restenosis of a blood vessel or having an injured blood vessel. The method comprises administering to the patient a vector encoding a Fat1 having an amino acid sequence at least 90% identical to SEQ ID NO:1 or SEQ ID NO:2, where the vector is capable of expressing the Fat1 in

cells of the patient, and wherein the Fat1 is capable of inhibiting growth and promoting migration of vascular smooth muscle cells (VSMC).

The invention is further directed to a method of treating an injured blood vessel in a patient. The method comprises administering a compound to the injured blood vessel, where the compound specifically binds to Fat1 and prevents the ability of the Fat1 to promote migration of vascular smooth muscle cells (VSMC).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a graph, a photograph of a western blot, and micrographs of stained tissue sections showing expression of Fat1 in normal and balloon-injured rat carotid arteries. Panel B shows results of a qPCR analysis of fat1 mRNA expression. Fat1 mRNA levels were corrected relative to gapdh mRNA levels, with day 0 (no injury) set=1. *, $P<0.05$, **, $P<0.01$ vs. day 0. Panel B shows the specificity of anti-Fat1 antiserum. Preimmune and anti-Fat1 immune rabbit sera (1:5000 dilutions) were tested by immunoblotting of replicate total RASMC protein extracts (20 μg/lane). The arrow indicates the Fat1 signal. Panel C shows an immunohistochemical analysis of Fat1 expression in arteries 0, 3, 7, and 14 days after injury, as indicated. Arrowheads indicate the internal elastic lamina, and double-ended arrows, the extent of the media. The neointima is the space between arrowhead and arrow. The orientation of all samples is similar, with an asterisk in the day 0 panel indicating the vessel lumen. The upper right panel shows a day 7 sample co-stained for Fat1 and PCNA; areas of increased Fat1 (arrow) and increased PCNA (arrowhead) staining are indicated. The lower right panel shows a day 7 sample in which Fat1 antiserum was omitted (no 1° ab). Scale bar, 100 μm.

FIG. **2** shows photographs of western blots showing a western analysis of Fat1 expression in RASMCs. Cells were serum-deprived, stimulated as described, and harvested for protein extraction at the indicated time points. Actin expression is shown as a loading reference. Data are representative of three independent experiments. Panel A shows induction by 10% FBS for 0-36 h prior to protein extraction. The blot was also probed for cyclin D1. Panel B shows induction by specific factors. Cells were stimulated with ATII, 10^{-6} mol/L, bFGF, 20 ng/mL, or PDGF-BB, 20 ng/mL.

FIG. **3** is photographs of western blots, fluorescent and light micrographs of cells, and graphs showing the effect of decreased Fat1 expression on VSMC migration. Panel A shows a western analysis of Fat1 specific and control siRNA efficacy, 48 h after transfection. Panel B shows a schematic diagram of IL2R-Fat1$_{IC}$. EC, extracellular; TM, transmembrane; IC, intracellular, with photomicrograph of subcellular localization of IL2R-Fat1$_{IC}$-3XFLAG in transfected cells stained with FITC-conjugated anti-FLAG antibody. Panel C shows MASMC migration 30 h after wounding of monolayer. Dashed lines indicate extent of initial denudation. The graphs shows quantitative analysis of MASMC migration after control (Ctl) and specific Fat1 siRNA transfection. For 10 matched fields, the area of the wounded monolayer covered by cells at the indicated timepoints was determined by planimetry using NIH Image, and distance migrated calculated according to the difference from time 0. *, $P<0.05$ vs. Ctl siRNA. Panel D shows results of a transwell migration assay of A7r5 cells transduced with the indicated retroviruses. Six fields were counted per condition, and the values were averaged for each filter. *, $P<0.05$ vs. other groups.

FIG. **4** is photographs of western blots and a graph showing the effect of decreased Fat1 expression on VSMC cell cycle progression. Panels A and B show a western analyses of Fat1 and cyclin D1 expression with control (Ctl) or Fat1-specific siRNAs. Panel A shows results when four distinct Fat1-specific siRNAs were transfected 48 h prior to protein extraction. Loading reference, α-tubulin. Panel B shows the efficacy of Ctl or Fat1-specific siRNA 7296 over time. Actin and O-catenin expression were also tested. Panel C shows the effect of Fat1 inhibition on DNA synthesis assessed by BrdU incorporation. Cells were transfected with Ctl or Fat1 siRNA, serum-deprived for 48 h, and stimulated with 10% FBS. BrdU incorporation was assessed as described in Materials and Methods. The graph depicts the means±S.E. of three independent experiments in which a total of 219-874 cells were counted each time for each group. *, $P<0.05$ vs. Ctl siRNA.

FIG. **5** is photographs of western blots and graphs showing the effect of IL2R-Fat1$_{IC}$ expression on VSMC growth. RV, IL2R, and IL2R-Fat1$_{IC}$ designate A7r5 or MASMCs transduced with the corresponding retroviral constructs. Panel A shows a western analyses of IL2R-Fat1$_{IC}$ expression in A7r5 and MASMC stable transfectants. A non-specific band (ns) near the IL2R-Fat1$_{IC}$ protein is indicated. The blots were also probed for cyclin D1 and actin. Panel B shows the effect of IL2R-Fat1$_{IC}$ on A7r5 cell growth. Cell number was calculated by CyQuant fluorescence assay by reference to a standard curve. Panel C shows the effect of IL2R-Fat1$_{IC}$ on DNA synthesis in A7r5 and MASMCs, evaluated by BrdU incorporation. *, $P<0.05$ vs. control.

FIG. **6** is fluorescence micrographs and photographs of western blots showing the co-localization and interaction of endogenous β-catenin and Fat1 in VSMCs. Panel A shows an immunofluorescence analysis of β-catenin (β-cat), Fat1, and areas of co-localization ("merge"). Nuclei were stained with DAPI as indicated. β-catenin and Fat1 co-localization at cell-cell junctions is indicated by an arrowhead. Scale bar (10 μm) applies to all panels. Panel B shows detail from the panels in A, showing staining for Fat1 (right micrograph), but not β-catenin (left), at the cellular free edge. Scale bar, 10 μm. Panel C shows the co-immunoprecipitation of endogenous β-catenin and Fat1. Cell lysates were incubated with antibodies specific for Fat1 (upper panels) or β-catenin (lower panels) or normal rabbit or mouse IgG, and the immunoprecipitated complexes were analyzed by western blot for Fat1 and β-catenin, as indicated.

FIG. **7** is a diagram and photographs of western blots showing the identification of β-catenin-interacting residues in the Fat1$_{IC}$ domain by co-immunoprecipitation of epitope-tagged proteins. Panel A is a schematic depiction of FLAG-tagged IL2R-Fat1$_{IC}$ deletion constructs. Fat1$_{IC}$ domains indicated: juxtamembrane (JM), FC1, FC2, carboxy-terminus (CT). Panel B shows a western analysis of immunoprecipitated protein complexes. The indicated constructs were transfected into 293T cells. Upper blots: after 24 h, total cellular protein (400 μg) was harvested and analysed by immunoprecipitation and western blotting (WB) with antibodies against the epitope tags, as indicated. Lower blots: protein input (7.5%). An analogous E-cadherin-derived construct (IL2R-E-cad$_{IC}$-FLAG) was used as a positive control.

FIG. **8** is fluorescence micrographs and photographs of western blots showing the effect of Fat1$_{IC}$ overexpression on β-catenin nuclear localization in VSMCs. Panel A shows immunofluorescence analysis of β-catenin subcellular localization in IL2R-GFP-RV (upper) and IL2R-Fat1$_{IC}$-GFP-RV (lower) transduced RASMCs. Cells were treated with LiCl (20 mmol/L) for 6 h, and then stained with anti-β-catenin antibody and DAPI. Transduced cells were identified by co-expressed GFP. Arrows indicate nuclear β-catenin signal of untransduced and transduced cells within each panel (see

text). Scale bar, 10 μm. Panel B shows western analysis of β-catenin in membrane (M), cytoplasmic (C), and nuclear (N) fractions extracted from IL2R-GFP-RV and IL2R-Fat1$_{IC}$-GFP-RV transduced A7r5 cells treated with LiCl, as above. The blot was probed for lamin A/C to assess fractionation and loading.

FIG. **9** is graphs and fluorescent micrographs showing the effect of altered Fat1 expression on β-catenin transcriptional activity in VSMCs. Panel A shows Topflash (TCF-luciferase reporter) activation. Topflash or Fopflash control was transfected into A7r5 cells along with expression constructs for β-catenin, IL2R-Fat1$_{IC}$, N-cadherin, and/or IL2R-E-cadherin$_{IC}$. The maximal reporter activity was set to 100. *, P<0.05, **, P<0.01, vs. activity with β-catenin alone. Panel B shows β-catenin localization in MASMCs transfected with control (Ctl, scrambled) or Fat1-specific (7296) siRNAs and stimulated with LiCl (20 mM) for 12 h. Panel C shows Topflash activity with decreased Fat1 expression. MASMCs transfected with the indicated siRNAs and the Topflash reporter were stimulated with LiCl (20 mM) for 12 h prior to assay for luciferase activity. *, P<0.05, vs. activity with Ctl siRNA. Panel D shows Cyclin D1 promoter activation. The cyclin D1 promoter-luciferase construct was transfected into A7r5 cells along with test constructs, as in A. *, P<0.05, **, P<0.01, vs. activity with β-catenin alone.

FIG. **10** is a photograph of a western blot, fluorescent micrographs, and a graph showing cleavage, localization, and activity of Fat1 cytoplasmic sequences in VSMC protein extracts. Panel A shows western analysis of A7r5 extracts transduced with IL2R-Fat1$_{IC}$ retrovirus. Total cellular protein was incubated at 37° C. for 15 min with or without proteinase inhibitors (inhib). Both full length (FL) Fat1 and the fusion protein (IL2R-Fat1$_{IC}$), are apparent with proteinase inhibition; only a single band (Fat1$_{IC}$*) of ~50 kD is seen without inhibition. Panel B shows subcellular localization of the FLAG-tagged Fat1 cytoplasmic domain with (Fat1$_{4189-4587}$) or without (Fat1$_{4201-4587}$) the putative NLS in transfected A7r5 cells. Anti-FLAG immunofluorescence and DAPI nuclear stains are shown. Panel C shows the effect of the NLS on Fat1$_{IC}$-mediated inhibition of β-catenin activation of the cyclin D1 promoter. Luciferase activity was assessed 24 h after transfection of A7r5 cells with the indicated expression constructs and the cyclin D1 promoter reporter. *, P<0.01 vs. activity with β-catenin alone.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have discovered that Fat1 regulates growth and migration of vascular smooth muscle cells (VSMCs). See Example. In particular, it was discovered that the complete Fat1 protein inhibits proliferation (i.e., growth) and promotes migration of VSMCs and the intracellular domain (i.e., cytoplasmic fragment) of Fat1 inhibits both proliferation and migration of VSMCs. These discoveries make useful various invention compositions and enable various therapeutic methods.

The invention is directed to an extracellular domain of a mammalian Fat1 (Fat1$_{EC}$), the extracellular domain comprising amino acids equivalent to amino acids 22-4174 of a wild-type mouse Fat1 having the amino acid sequence of SEQ ID NO:1, where the extracellular domain does not comprise the entire mammalian Fat1.

As used herein, a Fat1 is FAT tumor suppressor homolog 1 (Drosophila) having UniProtKB/TrEMBL entry Q9QXA3. The mouse Fat1 wild-type amino acid sequence is provided herein as SEQ ID NO:1 and the mouse wild-type Fat1 mRNA sequence is provided in Genbank accession no. NM_00_001081286. The human wild-type Fat1 amino acid sequence is provided herein as SEQ ID NO:2 and the human wild-type Fat1 in RNA sequence is provided in Genbank accession no. NM_005245.3. It is expected that any other wild-type mammalian Fat1 amino acid sequence could be determined by the skilled artisan. Such an amino acid sequence would be expected to be at least 85% identical to SEQ ID NO:1 and/or SEQ ID NO:2.

The Fat1 protein is made up of a signal peptide consisting of residues 1-21 of both SEQ ID NO:1 and SEQ ID NO:2; an extracellular domain consisting of resides 22-4174 of SEQ ID NO: 1 and 22-4181 of SEQ ID NO:2; a transmembrane domain consisting of residues 4175-4198 of SEQ ID NO:1 and 4182-4201 of SEQ ID NO:2; and an intracellular domain consisting of residues 4199-4598 of SEQ ID NO:1 and 4202-4588 of SEQ ID NO:2.

The Fat1 or fragments thereof claimed herein or used in the methods claimed herein includes mutants comprising amino acid deletions, substitutions or additions, provided the mutant inhibits proliferation (i.e., growth) and promotes migration of VSMCs and the intracellular domain (i.e., cytoplasmic fragment) of the mutant inhibits both proliferation and migration of VSMCs. Methods for determining these characteristics are provided, e.g., in the Example.

The invention Fat1$_{EC}$ can further comprise other amino acids or other compounds. Examples of additional amino acids includes the signal sequence, or a portion of the transmembrane domain. Examples of another compound that can usefully be bound to the invention Fat1$_{EC}$ is a fluorescent dye.

The invention Fat1$_{EC}$ is preferably a mouse Fat1 having an amino acid sequence at least 95% identical to SEQ ID NO:1. More preferably, the invention Fat1$_{EC}$ is a human Fat1 having an amino acid sequence at least 95% identical to SEQ ID NO:2. Most preferably, the invention Fat1$_{EC}$ is capable of inhibiting growth and promoting migration of vascular smooth muscle cells (VSMC).

When used for therapeutic purposes, the invention Fat1$_{EC}$ is preferably in a pharmaceutically acceptable carrier.

By "pharmaceutically acceptable" it is meant a material that (i) is compatible with the other ingredients of the composition without rendering the composition unsuitable for its intended purpose, and (ii) is suitable for use with subjects as provided herein without undue adverse side effects (such as toxicity, irritation, and allergic response). Side effects are "undue" when their risk outweighs the benefit provided by the composition. Non-limiting examples of pharmaceutically acceptable carriers include, without limitation, any of the standard pharmaceutical carriers such as phosphate buffered saline solutions, water, emulsions such as oil/water emulsions, microemulsions, and the like.

The above-described mutant Fat1$_{EC}$ can be formulated without undue experimentation for administration to a mammal, including humans, as appropriate for the particular application. Additionally, proper dosages of the compositions can be determined without undue experimentation using standard dose-response protocols.

Although the Fat1$_{EC}$ can be easily formulated for oral, lingual, sublingual, buccal, intrabuccal, rectal, or nasal administration, it is preferred that they be formulated for parenteral administration, such as for example, by intravenous, intramuscular, intrathecal or subcutaneous injection, since that is the most preferred route of administration of these proteins. Parenteral administration can be accomplished by incorporating the compounds into a solution or suspension. Such solutions or suspensions may also include sterile diluents such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol

or other synthetic solvents. Parenteral formulations may also include antibacterial agents such as for example, benzyl alcohol or methyl parabens, antioxidants such as for example, ascorbic acid or sodium bisulfite and chelating agents such as EDTA. Buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose may also be added. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

The invention is also directed to an intracellular domain of a mammalian Fat1 ($Fat1_{IC}$), the intracellular domain comprising amino acids equivalent to amino acids 4199-4598 of a wild-type mouse Fat1 having the amino acid sequence of SEQ ID NO:1, where the extracellular domain does not comprise the entire mammalian Fat1.

Preferably, the invention $Fat1_{IC}$ is a mouse Fat1 having an amino acid sequence at least 95% identical to SEQ ID NO:1. More preferably, the Fat1 is a human Fat1 having an amino acid sequence at least 95% identical to SEQ ID NO:2. Most preferably, the $Fat1_{IC}$ is capable of inhibiting both growth and migration of vascular smooth muscle cells (VSMC).

The $Fat1_{IC}$ here can further usefully be fused to an amino acid sequence to make a fusion protein capable of localizing the $Fat1_{IC}$ to a cell membrane. See Example. Preferably, the fusion amino acid sequence is a transmembrane region of an interleukin 2 receptor α-chain.

When used for therapeutic purposes, the invention $Fat1_{EC}$ is preferably in a pharmaceutically acceptable carrier, as described above.

The above-described invention $Fat1_{EC}$ and $Fat1_{IC}$ can also be provided as a vector for transfection of mammalian cells. Thus, the invention is additionally directed to a vector comprising a nucleic acid sequence encoding the above-described $Fat1_{EC}$, where the vector is capable of expressing the $Fat1_{EC}$ in a mammalian cell.

Also, the invention is directed to a vector comprising a nucleic acid sequence encoding the above-described $Fat1_{IC}$, wherein the vector is capable of expressing the $Fat1_{IC}$ in a mammalian cell.

The above $Fat1_{EC}$ can be applied to a vascular stent. Such a stent is useful for therapeutic applications (further discussed below). The ability to inhibit growth of VSMCs but not endothelial cells is useful for insertion into a blood vessel or vein where there is a risk for restenosis, since having an intact endothelium reduces the risk for thrombosis when compared to the common drug-eluting stents, which also inhibit endothelial cells. Thus, the invention is further directed to a vascular stent coated with the above-described $Fat1_{EC}$ that is capable of inhibiting growth and promoting migration of VSMC.

The benefits of the Fat_{EC}-coated stents discussed immediately above can also be achieved by instead coating the stent with a Fat1 ligand that activates Fat1. Thus, the invention is also directed to vascular stents coated with a Fat1 ligand that activates Fat1. The Fat1-activating ligand can be identified by, e.g., screening a library of compounds for the ability to activate Fat1. Preferably the library comprises antibodies (e.g., monoclonal antibodies or phage display antibodies). A preferred Fat1 ligand is an antibody, e.g., identified by that screening procedure.

The above compositions are particularly useful in methods of treating patients that have undergone angioplasty, bypass surgery or other similar procedures. In one aspect of therapeutic methods, the invention is directed to a method of treating a patient at risk for restenosis of a blood vessel, the method comprising inserting the above-described vascular stent coated with the $Fat1_{EC}$ into the blood vessel at the site of the risk. Preferably here, the blood vessel is an artery, most preferably a coronary artery.

The invention is also directed to a method of treating an injured blood vessel in a patient. The method comprises administering the above-described $Fat1_{IC}$ or the above-described $Fat1_{IC}$ vector to the injured blood vessel in a manner sufficient to treat the injured blood vessel. Where the vector is used, it is administered such that it can transfect VSMC cells in the blood vessel. In these methods, the administered $Fat1_{IC}$, or the $Fat1_{IC}$ synthesized from the vector, is useful in preventing restenosis due to the ability of the $Fat1_{IC}$ to inhibit VSMC proliferation. Optionally, the $Fat1_{IC}$ can further comprise the above-described amino acid sequence that targets the $Fat1_{IC}$ to the cell membrane. Preferably, the blood vessel is an artery or vein used in a coronary artery bypass surgery.

The invention is additionally directed to a method of treating a patient at risk for restenosis of a blood vessel or having an injured blood vessel. The method comprises administering to the patient a vector encoding a Fat1 having an amino acid sequence at least 90% identical to SEQ ID NO:1 or SEQ ID NO:2, where the vector is capable of expressing the Fat1 in cells of the patient, and wherein the Fat1 is capable of inhibiting growth and promoting migration of vascular smooth muscle cells (VSMC). The Fat1 expressed from the vector would inhibit growth and facilitate migration of VSMCs. Preferably here the Fat1 is a human Fat1 having an amino acid sequence at least 95% identical to SEQ ID NO:2. More preferably, the Fat1 is a human Fat1 having an amino acid sequence at least 99% identical to SEQ ID NO:2. The vector is also preferably administered to the patient during or after coronary artery bypass surgery. Alternatively, the vector is administered to the patient during or after angioplasty.

The invention is further directed to a method of treating an injured blood vessel in a patient. The method comprises administering a compound to the injured blood vessel, where the compound specifically binds to Fat1 and prevents the ability of the Fat1 to promote migration of vascular smooth muscle cells (VSMC). Such an application is useful to limit migration and oppose VSMC accumulation in arterial neointima.

In some aspects of these methods, the compound comprises an antibody binding site. Preferably, the compound is an antibody that has previously been shown to inhibit Fat1 activity. Such antibodies can be prepared without undue experimentation. Alternatively, the compound is an aptamer. These methods are most preferably used where the blood vessel is an artery or vein used in a coronary artery bypass surgery.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims, which follow the examples.

Example

The Fat1 cadherin integrates vascular smooth muscle cell growth and migration signals.

Example Summary

This Example is based on the publication Hou et al., 2006. The significance of cadherin superfamily proteins in vascular smooth muscle cell (VSMC) biology is undefined. Described here are recent studies of the Fat1 protocadherin. Fat1 expression in VSMCs increases significantly after arterial injury or growth factor stimulation. Fat1 knockdown decreases VSMC migration in vitro, but surprisingly, enhances cyclin D1 expression and proliferation. Despite limited similarity to classical cadherins, the Fat1 cytoplasmic domain ($Fat1_{IC}$) interacts with β-catenin, inhibiting both its nuclear localization and transcriptional activity. Fat1 undergoes cleavage and $Fat1_{IC}$ species localize to the nucleus; however, inhibition of the cyclin D1 promoter by truncated $Fat1_{IC}$ proteins corresponds to their presence outside the nucleus, which argues against repression of β-catenin-dependent transcription by nuclear $Fat1_{IC}$. These findings extend recent observations about Fat1 and migration in other cell types, and demonstrate for the first time its anti-proliferative activity and interaction with O-catenin. Because it is induced after arterial injury, Fat1 may control VSMC functions central to vascular remodeling by facilitating migration and limiting proliferation.

Introduction

These studies show that Fat1 expression increases after injury of the rat carotid artery, and is positively regulated in cultured VSMCs by several factors that promote cell proliferation and migration. Interestingly, knockdown of Fat1 expression limits VSMC migration, but enhances VSMC growth. This anti-proliferative effect of Fat1 appears to be mediated by $Fat1_{IC}$ sequences, since expression of a fusion protein containing the $Fat1_{IC}$ inhibits cyclin D1 expression and cell growth. Moreover, the $Fat1_{IC}$ can interact with O-catenin, prevent its nuclear translocation, and limit its transcriptional activity on both synthetic and native β-catenin-responsive promoters, including that of cyclin D1, a known target of canonical Wnt signaling. These findings point to an integrative role for Fat1 in regulation of critical VSMC activities, in which it promotes migration and limits both canonical Wnt signaling and VSMC growth in the remodeling artery.

Results

Expression of Fat1 Increases after Arterial Injury.

fat1 mRNA expression was quantified by quantitative PCR (qPCR) of cDNA samples from normal and injured rat carotid arteries. Compared to uninjured arteries, fat1 mRNA expression was ~8.5-, 13.0-, and 3.9-fold higher than control at 3, 7, and 14 days after injury, respectively (FIG. **1**A).

To localize Fat1 protein expression in injured arteries, we characterized rabbit antisera raised against a GST-$Fat1_{IC}$ immunogen. Immunoblotting of VSMC lysates with one such antiserum, but not preimmune serum, yielded a single high molecular weight band of ~500 kD, in accord with the predicted size of full length Fat1 (FIG. **1**B). Further specificity was demonstrated in RNA interference experiments directed against multiple separate targets in the mouse Fat1 sequence (FIGS. **3** and **4**). The antiserum was then used for immunohistochemical studies. As shown in FIG. **1**C, prominent Fat1 staining appeared in the media 3 days after injury, while at 7 days and 14 days after injury, Fat1 staining was less evident in the media, but clearly present in the developing neoinitima. Western analysis of Fat1 expression in the carotid artery injury model, like our qPCR findings, showed a clear induction after injury (data not shown). To correlate Fat1 expression with the proliferative status of specific cells, we co-stained sections for Fat1 and the proliferation marker PCNA. While some cells appeared positive for both, we also noted some spatial separation of the signals, particularly evident in areas with limited neointimal formation, which showed prominent Fat1 staining without PCNA (FIG. **1**C, upper right). The latter observation raised the possibility that, despite its overall induction after injury, increased Fat1 expression might have negative effects on VSMC growth in vivo (FIG. **1**C, upper right).

Serum and Growth Factors Induce Fat1 Expression in VSMCs.

To identify factors that might contribute to Fat1 induction after arterial injury, its expression was characterized in primary cultured VSMCs. Quiescent rat aortic smooth muscle cells (RASMCs) (time 0 h) were treated with 10% FBS for 2, 6, 12, 18, 24, and 36 h, and the level of Fat1 protein was determined by Western analysis. The Fat1 signal increased strongly between 2 and 12 h and remained elevated through 36 h (FIG. **2**A). To assess cell cycle status, we also checked cyclin D1 expression in these lysates. Interestingly, Fat1 induction preceded the increase of cyclin D1, a mediator of progression through the G1 phase of the cell cycle (FIG. **2**A).

We then assessed Fat1 expression in response to several factors known to affect the vascular response to injury. Western analyses showed that expression of Fat1 increased in response to Angiotensin II (ATII), basic FGF (bFGF), and PDGF-BB (FIG. **2**B). Increased Fat1 expression was apparent by 2 h and sustained at high levels from 12 to 36 h after stimulation with each of these factors. Thus, Fat1 expression is regulated consistently and strongly by multiple factors known to promote VSMC growth and migration.

Inhibition of Fat1 Expression Limits VSMC Migration.

Two recent studies have described a role for Fat1 in regulation of epithelial cytoskeletal actin dynamics, planar polarity, and migration, mediated through interactions of the Fat1 cytoplasmic domain with proteins of the Ena/VASP family (Moeller et al., 2004; Tanoue and Takeichi, 2004). Fat1 induction by known VSMC chemotactic factors (FIG. **2**) suggested that Fat1 might also be involved in VSMC migration. To test this and other potential Fat1 functions, we developed reagents to effectively manipulate Fat1 expression. Transfection of mouse aortic smooth muscle cells (MASMCs) with Fat1 specific small interfering RNAs (siRNAs), but not scrambled or mismatch derivatives, resulted in significantly decreased levels of Fat1 protein (FIG. **3**A). To isolate and augment signals mediated by the $Fat1_{IC}$, we generated a cDNA construct, IL2R-$Fat1_{IC}$, in which the entire Fat1 cytoplasmic domain was fused to the extracellular domain and transmembrane region of the interleukin 2 receptor x-chain (IL2R), with or without a C-terminal FLAG epitope tag (FIG. **3**B). Subcellular localization of this fusion protein was tested in 3T3 cells, which do not express detectable Fat1, and A7r5 VSMCs, which express moderate amounts of endogenous Fat1; both transfected 3T3 and A7r5 cells showed an appropriate cell surface signal when stained with anti-FLAG epitope antibody (FIG. **3**B and data not shown). Cell migration in monolayers treated with specific Fat1 siRNA was modestly but significantly decreased compared with control siRNA (FIG. **3**C), which indicates that Fat1 expression is required for optimal VSMC migration. Surprisingly, we also found decreased migration of VSMCs expressing the IL2R-$Fat1_{IC}$ protein in a Transwell assay using FBS as a stimulant in the lower chamber (FIG. **3**D). Both expression of Ena/VASP proteins in

VSMCs, and the ability of the IL2R-Fat1$_{IC}$ protein to interact with these signaling intermediates was confirmed (data not shown). It is surmised that although the IL2R-Fat1$_{IC}$ construct may increase intracellular Fat1 signaling, it also dissociates Fat1 extracellular interactions from this intracellular signaling, and thus interferes with directional migration. Altogether, these findings indicate that Fat1 promotes VSMC migration; it is likely that, as described in epithelial cells, interactions with Ena/VASP proteins link Fat1 expression to VSMC cytoskeletal actin reorganization, polarization, and migration.

Inhibition of Fat1 Expression Promotes VSMC Growth.

In addition to increased migration, the VSMC response to injury is characterized by cell cycle entry and increased proliferation (Clowes et al., 1983a). To evaluate how Fat1 induction after injury might affect VSMC growth, the effect of Fat1 knockdown on expression of cyclin D1 (a marker of cell cycle activation) was tested. Four distinct mouse Fat1 siRNA duplexes attenuated endogenous Fat1 levels in MASMCs; with each duplex, a significant increase in cyclin D1 expression over control levels was also observed (FIG. **4**A and data not shown). The similarity of effect achieved by multiple distinct siRNAs argues strongly that increased cyclin D1 expression results from decreased Fat1, and not an off-target effect. The duration of Fat1 inhibition was more than 90% at 2 and 3 days after transfection, with persistent and strong inhibition still apparent after 6 days (FIG. **4**B). Decreased Fat1 expression corresponded to increased cyclin D1 signal at each time point (2.0-2.5-fold increase of cyclin D1/actin ratio vs. control), suggesting that endogenous Fat1 exerts a tonic inhibitory effect on cyclin D1 expression (FIG. **4**B). The level of total β-catenin in these cells, by contrast, showed little change.

The effect of Fat1 knockdown on DNA synthesis was also examined. Cells were transfected with Fat1 or control siRNA, and then serum deprived for 48 h prior to stimulation with 10% FBS and evaluation of BrdU incorporation. In Fat1 knockdown cultures, the fraction of BrdU positive cells was significantly higher than in control siRNA cells (52±7% vs. 30±8%, $P<0.05$) (FIG. **4**C). These findings indicate that decreased Fat1 expression promotes cell cycle progression and DNA synthesis in VSMCs.

The Fat1$_{IC}$ is Sufficient to Inhibit VSMC Growth.

Classical cadherins interact with intracellular signaling pathways through their cytoplasmic domains (Wheelock and Johnson, 2003a). To establish cell populations differing primarily in their expression of the Fat1$_{IC}$, the IL2R (without cytoplasmic domain) and IL2R-Fat1$_{IC}$ constructs were transferred into the GFP-RV retroviral vector (Ranganath et al., 1998). Viral supernatants were produced and used to transduce A7r5 and primary MASMCs. Additional control cells, denoted RV, were produced using the unmodified GFP-RV vector. Western analysis confirmed IL2R-Fat1$_{IC}$ expression in A7r5 and MASMCs (FIG. **5**A). Interestingly, endogenous cyclin D1 levels were lower in both A7r5 and MASMCs expressing IL2R-Fat1$_{IC}$ (FIG. **5**A). In cell growth assays over 7 days, A7r5 cells expressing IL2R showed no significant change from control RV cells, but decreased cell numbers were evident in the IL2R-Fat1$_{IC}$ at all timepoints after 3 days (FIG. **5**B). In addition, both A7r5 and MASMCs expressing the IL2R-Fat1$_{IC}$ construct showed significantly lower fractions of BrdU-positive nuclei, indicating that this decrease in cell number reflected growth inhibition rather than decreased survival (FIG. **5**C). Fat1 and β-Catenin co-localize and interact in VSMCs. In epithelial cells, classical cadherins such as E-cadherin regulate Wnt signaling activity by physically associating with β-catenin at points of cell-cell contact (Nathke et al., 1994). The sequences, interacting proteins, and functions of protocadherin cytoplasmic domains are typically thought to be divergent from those of the classical cadherins (Yagi and Takeichi, 2000), and Fat1 is not regarded as part of the classical cadherin system (Tanoue and Takeichi, 2005). Nevertheless, we found that the Fat1$_{IC}$ has growth inhibitory activity, and that expression of cyclin D1, a known target of the canonical Wnt signaling pathway, correlated negatively with Fat1$_{IC}$ expression. Together these findings suggested that growth inhibition by Fat1 might involve β-catenin. In our immunofluorescent analyses of RASMCs (FIG. **6**), Fat1 localized to both cell-cell junctions and cellular free edges, while β-catenin was concentrated at sites of cell-cell contact. By two color immunofluorescence analysis, we found areas along cell-cell junctions where the two signals overlapped (FIG. **6**A). This overlap did not include the cellular free edges, where Fat1 alone was seen (FIG. **6**B).

Junctional β-catenin and Fat1 have been identified in epithelial cells that display apical-basal polarity, but it is thought that the two proteins occupy distinct domains, with β-catenin at apical adherens junctions and Fat1 at basolateral points of cell-cell contact (Tanoue and Takeichi, 2004; Tanoue and Takeichi, 2005). VSMCs are non-polarized (Muller and Gimbrone, 1986), so this model of apical-basal domain specialization may not apply. To test directly if Fat1 and O-catenin can interact at physiologic levels of expression in VSMCs, endogenous Fat1 was immunoprecipitated. Recovery of O-catenin was also determined. Both this assay and reciprocal co-immunoprecipitations of β-catenin followed by immunoblotting for Fat1 demonstrated interaction of the two proteins (FIG. **6**C). This finding suggests that the non-polarized nature of VSMCs allows for protein-protein interactions not found in polarized cell types such as epithelial cells. Further immunoblotting of Fat1 immunoprecipitates with a pan-cadherin antibody did not reveal associated (classical) cadherins that might associate with both Fat1 and β-catenin (data not shown).

To characterize the Fat1-β-catenin interaction further, co-immunoprecipitation assays were used in co-transfected 293T cells to map the sequences required for interaction. A series of constructs bearing deletions within the Fat1$_{IC}$ portion of the IL2R-Fat1$_{IC}$-3XFLAG were generated (FIG. **7**A). IL2R-E-cadherin$_{IC}$-3XFLAG and IL2R-3XFLAG (containing no Fat1 sequences) constructs served as positive and negative controls, respectively. The expression of Myc-tagged β-catenin and FLAG-tagged fusion proteins was confirmed, as was immunoprecipitation of transfected Myc-tagged β-catenin (FIG. **7**B, lower panels). Interaction of β-catenin with the IL2R-Fat1$_{IC}$-3XFLAG derivatives was assessed by immunoblotting with FLAG antibody (FIG. **7**B, upper panel). A robust FLAG signal was obtained with the IL2R-Fat1$_{IC}$-3XFLAG construct containing the complete Fat1$_{IC}$ domain and with derivatives 1, III, and V. Weaker signals were seen with constructs II and IV, which lack the FC1 and both FC1 and FC2 domains, respectively. While these findings based on overexpressed proteins must be interpreted with caution, they suggest that β-catenin interacts with the Fat1$_{IC}$ principally through the FC1 domain, but leave open the possibility that the FC2 domain or additional sequences also contribute to the interaction. Interestingly, the E-cadherin-based positive control yielded a comparatively strong band, despite input of substantially less protein.

Expression of the Fat1$_{IC}$ Affects β-Catenin Cellular Distribution and Transcriptional Activity.

As noted above, changes in Fat1 or Fat1$_{IC}$ expression affected expression of a O-catenin target gene, cyclin D1, but had little effect on overall β-catenin levels (FIG. **4**B). Having

found evidence for co-localization and interaction of β-catenin and Fat1 in VSMCs, it was postulated that Fat1 might be acting like a classical cadherin to affect the subcellular localization and activity of β-catenin. This was first examined using immunocytochemistry. Expression plasmids encoding IL2R or IL2R-Fat1$_{IC}$ were introduced into VSMCs, which were subsequently treated with LiCl (20 mM) for 6 h to activate Wnt signaling and promote nuclear translocation of β-catenin (Hedgepeth et al., 1997). The intensity of nuclear β-catenin staining did not appear to be affected by expression of IL2R (arrows, FIG. **8**A, upper panel). In contrast, nuclear accumulation of β-catenin appeared decreased in the IL2R-Fat1$_{IC}$-expressing cells (arrows, FIG. **8**A, lower panel), as compared with untransfected cells. To assess this effect in a more quantitative way, the distribution of β-catenin was determined in the membrane, cytoplasmic, and nuclear fractions of IL2R-GFP-RV and IL2R-Fat1$_{IC}$-GFP-RV transduced VSMC cultures treated with LiCl. As shown in FIG. **8**B, immunoblotting showed a relative decrease in nuclear β-catenin accumulation in cells expressing IL2R-Fat1$_{IC}$, as compared with those expressing IL2R (respective nuclear β-catenin/lamin A/C ratios 0.8 (IL2R-Fat1$_{IC}$) vs 1.65 (IL2R)).

To assess further the functional significance of the Fat1-β-catenin interaction in VSMCs, we tested the effect of Fat1$_{IC}$ overexpression on β-catenin-mediated transcription. A7r5 cells were co-transfected with β-catenin and/or IL2R-Fat1$_{IC}$, along with the TCF-luciferase reporter construct Topflash or its negative control, Fopflash (FIG. **9**A). Topflash reporter activity reflects activation of the canonical Wnt signaling pathway, β-catenin nuclear translocation, and formation of TCF/β-catenin heterodimers; Fopflash contains mutated TCF binding sites and serves as a control for non-specific activation (Korinek et al., 1997). A full-length N-cadherin cDNA and the IL2R-E-cadherin$_{IC}$ construct were also tested as controls. Specific activation of Topflash by β-catenin was ~10-fold above basal levels, and the three test constructs all inhibited this activation significantly. Interestingly, the inhibition due to both IL2R-Fat1$_{IC}$ (40%) and N-cadherin (55%) was less complete than that resulting from co-transfection of IL2R-E-cadherin$_{IC}$, which abolished all β-catenin-mediated transactivation. We also evaluated the effect of decreased Fat1 expression. Immunocytochemistry of LiCl-stimulated MASMCs suggested a relative enhancement of nuclear O-catenin staining in Fat1-depleted cells (FIG. **9**B). To assess this observation more quantitatively, we transfected MASMCs first with control or Fat1-specific siRNA and then with the Topflash reporter. As shown in FIG. **9**C, LiCl-stimulated TCF/β-catenin transcriptional activation was ~30% higher in Fat1 knockdown cells compared with control.

As shown in FIGS. **4** and **5**, cyclin D1 levels varied inversely with the level of Fat1$_{IC}$ expression. The cyclin D1 promoter is a known transcriptional target of Wnt signaling and activated TCF/β-catenin complexes (Shutman et al., 1999; Tetsu and McCormick, 1999), so we postulated that Fat1$_{IC}$ might also inhibit the native cyclin D1 promoter. VSMCs were co-transfected with β-catenin and/or IL2R-Fat1$_{IC}$, along with the cyclin D1 promoter-luciferase reporter construct (Herber et al., 1994). N-cadherin and the IL2R-E-cadherin$_{IC}$ fusion protein were also tested. Most of the β-catenin-mediated activation of the cyclin D1 promoter reporter was eliminated by IL2R-Fat1$_{IC}$ or N-cadherin expression (FIG. **9**D). Consistent with the Topflash results, IL2R-E-cadherin$_{IC}$ was more effective, as it decreased promoter activity to a level below baseline.

Inhibition of β-Catenin Activity Depends on Extranuclear Localization of the Fat1$_{IC}$.

Fat1 is a type I transmembrane protein, and immunofluorescence studies with antiserum specific for Fat1$_{IC}$ sequences showed expression at the cell surface, as expected (FIG. **6**). We also noted consistent signals in the cell nucleus with this antiserum. This observation, together with a recent report of localization of Fat1 cytoplasmic sequences to the nucleus (Magg et al., 2005), raised the possibility that inhibition of β-catenin by Fat1 might result from a nuclear (transcriptional repressor) function of a cleaved Fat1$_{IC}$ fragment, rather than sequestration of O-catenin outside the nucleus. Indeed, incubation without proteinase inhibitors of extracts of A7r5 cells expressing both native Fat1 and the IL2R-Fat1$_{IC}$ fusion protein showed the disappearance of these full length proteins and rapid appearance of a single, relatively stable species of ~50 kD (FIG. **10**A). Because the N-terminus of this cleaved product is not yet defined, we designate it as Fat1$_{IC}$*; its apparent size in SDS-PAGE suggests that it contains most, if not all, of the ~400 aa Fat1$_{IC}$ domain.

Like human Fat1$_{IC}$ (Magg et al., 2005), the mouse Fat1$_{IC}$ contains a potential nuclear localizing sequence (NLS) (RKMISRKKKR) near its N-terminus. The effect of this sequence on Fat1$_{IC}$ localization was tested by immunocytochemical analysis of A7r5 cells transfected with FLAG-tagged expression constructs that retain (Fat1$_{4189-4587}$) or exclude (Fat1$_{4201-4587}$) the NLS motif. Fat1$_{4189-4587}$ localized almost exclusively to the nucleus, while Fat1$_{4201-4587}$ was apparent in the nucleus and prominent throughout the cytoplasm (FIG. **10**B).

To evaluate these findings in the context of Fat1-mediated VSMC growth inhibition, these Fat1$_{IC}$ derivatives were tested for effects on cyclin D1 promoter activity. The IL2R-Fat1$_{IC}$ fusion protein yielded significant inhibition of β-catenin-mediated cyclin D1 promoter activation (FIG. **9**D, above); Fat1$_{4201-4587}$, but not Fat1$_{4189-4587}$, retained this inhibitory effect (FIG. **10**C). Both Fat1$_{4201-4587}$ and Fat1$_{4189-4587}$ are present in the nucleus, but the former has a cytoplasmic distribution not shared by Fat1$_{4189-4587}$; hence, we attribute this inhibitory effect on β-catenin to the extranuclear presence of Fat1$_{4201-4587}$.

Discussion

Fat1 is expressed widely during mouse and rat development (Cox et al., 2000; Ponassi et al., 1999), notably in areas with high levels of cellular proliferation. Although in situ hybridization of rat embryos demonstrated expression of fat1 mRNA in the developing aortic outflow tract (Ponassi et al., 1999), the significance of Fat1 in vascular tissues has not been explored previously.

We found relatively low expression of Fat1 in normal adult rat carotid arteries, and substantially increased levels during the first few days after injury (FIG. **1**A). Immunohistochemical analyses (FIG. **1**C) showed prominent Fat1 staining first in the injured arterial media, and subsequently in the neointima, a pattern of expression similar to that of VSMC proliferation in this model (Clowes et al., 1983b). Interestingly, areas of attenuated neointimal formation showed prominent Fat1 and decreased PCNA staining, providing an initial suggestion that Fat1 might act to limit VSMC proliferation in vivo (FIG. **1**C). Nevertheless, Fat1 levels in cultured VSMCs increased in response to serum and several factors known to promote VSMC activation and neointimal formation, including ATII (Powell et al., 1990), PDGF-BB (Ferns et al., 1991), and bFGF (Lindner and Reidy, 1991) (FIG. **2**). This expression pattern contrasts with that described for N-cadherin, which decreases after stimulation of VSMC with serum or PDGF-

BB (Uglow et al., 2003), and that of R-cadherin, which decreases substantially in the first few days after injury (Slater et al., 2004).

To evaluate how induction of this very large protocadherin might affect the response to vascular injury, we tested the effect of Fat1 on VSMC migration and proliferation, two of the key cellular functions activated in this setting. Both loss of Fat1 expression and expression of the IL2R-Fat1$_{IC}$ fusion protein attenuated VSMC migration (FIG. **3**). In the context of recent reports regarding Fat1 function in epithelial cells (Moeller et al., 2004; Tanoue and Takeichi, 2004), these findings suggest that increased Fat1 expression facilitates VSMC migration by providing directional cues and stimulating actin cytoskeletal remodeling through its interactions with proteins of the Ena/VASP family. Together with the Fat1 knockdown results, inhibition of migration by the IL2R-Fat1$_{IC}$ fusion protein suggests that dissociation of Fat1 extracellular interactions from Fat1$_{IC}$-mediated intracellular signaling interferes with directional migration.

Despite the induction of Fat1 in the proliferative phase after injury and in response to growth factor stimulation of cultured cells, our results in both loss- and gain-of-function studies (FIGS. **4**, **5**) suggest that Fat1 opposes VSMC proliferation. Loss of growth suppression resulting in imaginal disc overgrowth in *Drosophila* led to identification of Fat (Mahoney et al., 1991), the founding member of the cadherin subfamily that includes mammalian Fat1. While recent analyses indicates that mammalian Fat1 is more closely related to *Drosophila* Ftl (Castillejo-Lopez et al., 2004) than to Fat, a growth regulatory function has yet to be described for Ftl. Altered growth characteristics were also not identified in mouse Fat1−/− neural progenitors and embryonic skin (Ciani et al., 2003). Thus, our findings in VSMCs may reflect cell type-specific differences in the expression of cadherins or other protocadherins functionally redundant with Fat1, or differences in the level of β-catenin expression. In either case, the results of Fat1 knockdown studies indicate that in VSMCs, endogenous levels of Fat1 expression are sufficient to limit cyclin D1 expression (FIG. **4**) and β-catenin-mediated transcription (FIG. **9**), while our gain-of-function studies (FIG. **5**) suggest that decreased cyclin D1 expression and cell growth are likely physiologic consequences of Fat1 induction. Cyclin D1, a known TCF/β-catenin target gene (Shtutman et al., 1999; Tetsu and McCormick, 1999), plays a critical role in regulation of G1 phase progression and G1/S cell cycle transition (Jiang et al., 1993; Resnitzky et al., 1994), and the level of its expression is closely controlled. Increased Fat1 expression in response to injury probably acts to slow VSMC proliferation, at least in part by decreasing cyclin D1 expression.

Signaling by classical cadherins has been studied extensively, but the mechanisms of protocadherin signaling are not well understood. The intracellular portion of Fat1 shows limited similarity to classical cadherin cytoplasmic domains, with 30 of 137 (22%) residues matching consensus in the FC1 domain and 28 of 84 (33%) residues matching consensus in the FC2 domain (Dunne et al., 1995). Although Tanoue and Takeichi described partial co-localization of Fat1 and β-catenin in immortalized epithelial cell lines, they found more O-catenin in apical lateral cell contacts and more Fat1 in basal lateral cell contacts (Tanoue and Takeichi, 2004), and concluded that Fat1 does not participate in the classical cadherin system (Tanoue and Takeichi, 2005). Interestingly, these findings are consistent with the observation that in polarized epithelial cells, complexes forming between adjacent cells vary in composition according to their apical vs. basal position (Johnston and Gallant, 2002). Thus our findings in VSMCs, which are morphologically and biochemically non-polarized (Muller and Gimbrone, 1986), may differ because of the lack of apical-basal specialization in this cell type. In immunocytochemical studies, we found that β-catenin and Fat1 co-localized in a junctional pattern at points of contact between VSMCs (FIG. **5**); Fat1 staining was also observed at cellular free edges, while O-catenin was not.

It is believed that a physical interaction between endogenous Fat1 and O-catenin has not been demonstrated previously. Clear evidence was found that these proteins interact at physiologic levels of expression. Transfection studies with the IL2R-Fat1$_{IC}$ fusion protein indicated that, despite limited similarity to the β-catenin-interacting domains of classical cadherins, the Fat1$_{IC}$ domain was sufficient for this interaction (FIG. **7**). While mapping studies suggested that the Fat1 FC1 domain was most important for the β-catenin-Fat1 interaction, deletion of other domains within the Fat1$_{IC}$ also decreased the amount of protein co-immunoprecipitation, indicating that sequences both within and outside of the relatively conserved FC1 and FC2 domains may contribute to β-catenin-Fat1 interaction. Interestingly, the FC1 domain corresponds to the area of greatest similarity (54/196 aa identity, (27%)) with the *Drosophila* Ftl cytoplasmic domain; its role in the O-catenin-Fat1 interaction described here suggests that Ftl may be capable of interaction with armadillo, the *Drosophila* homologue of β-catenin.

The IL2R-Fat1$_{IC}$ chimera allowed functional analyses without confounding effects attributable to increased expression of the Fat1 extracellular domain. Expression of IL2R-Fat1$_{IC}$, but not a control protein lacking the Fat1$_{IC}$ domain, decreased nuclear translocation of O-catenin (FIG. **8**), and inhibited β-catenin transactivation of both synthetic (Topflash) and native (cyclin D1) TCF-dependent promoters (FIG. **9**). Although we found evidence of Fat1 cleavage resulting in a Fat1$_{IC}$* fragment that may localize to the nucleus (FIG. **10**), only a defined Fat1$_{IC}$ fragment lacking the NLS (aa 4189-4198) reproduced the inhibitory effect of the IL2R-Fat1$_{IC}$ fusion protein. This result suggests that inhibition of β-catenin transcriptional activity is mediated by Fat1$_{IC}$ outside the nucleus, and is not due to Fat1$_{IC}$ peptides in the nucleus. Thus, it remains to be determined if cleavage and nuclear translocation of Fat1$_{IC}$ underlies a specific function, perhaps as a chaperone or transcriptional regulator, or if it is important as a means to inactivate Fat1-mediated inhibition of β-catenin. Our studies to date indicate that the interaction of Fat1 cytoplasmic sequences with β-catenin has consequences for overall regulation of VSMC growth. The underlying mechanism appears similar to that described for classical cadherin-mediated sequestration of β-catenin in epithelial cells (Orsulic et al., 1999), but in the case of the protocadherin Fat1, this mechanism may be operative only in non-polarized cells such as VSMCs.

These findings suggest that increased expression of Fat1 after vascular injury facilitates migration and opposes proliferation of VSMCs. The former effect likely involves Fat1 interaction with Ena/VASP proteins, as described in other cell types (Moeller et al., 2004; Tanoue and Takeichi, 2004), while the latter effect relies on decreased nuclear accumulation of β-catenin (this study). Interestingly, Fat1$_{IC}$ interaction with, and inhibition of O-catenin both appeared less robust than that observed with classical cadherin sequences (FIGS. **7**, **9**), suggesting that Fat1 may be less efficient than the classical cadherins at sequestering β-catenin. Fat1 induction after injury and by growth factors contrasts with the expression pattern of other cadherins found in VSMCs. Together, these observations suggest that Fat1 may guide VSMC migration while remaining relatively permissive of growth in settings when VSMC proliferation is necessary for vascular repair.

Drosophila Ftl is thought to use its exceptionally large extracellular domain to promote epithelial cell separation during formation of tubular organs in embryogenesis (Castillejo-Lopez et al., 2004); it may be that mammalian Fat1, by virtue of its similar structure, may expedite circumferential distribution of VSMCs around the injured artery. Altogether, it is tempting to speculate that Fat1 limits VSMC proliferation while providing directional migration cues important during vascular remodeling, providing an integrative function that may oppose the formation of hyperproliferative cellular clusters. Finally, though expression of Fat1 in human vascular disease has not yet been evaluated, it is possible that loss of Fat1-mediated negative regulation could contribute to VSMC hyperplastic syndromes such as restenosis, transplant arteriopathy, or vein graft disease.

Materials and Methods

Rat Carotid Artery Balloon Injury.

All procedures were in accordance with institutional guidelines. The rat carotid artery balloon injury model was implemented as described (Sibinga et al., 1997). Briefly, male Sprague-Dawley rats (20 in total, Zivic-Miller) weighing 350 to 400 g were anesthetized with ketamine (40 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was denuded of endothelium and stretched by three passages of a 2F embolectomy catheter according to standard protocols. At 3, 7 and 14 days after injury, animals were reanesthetized and killed, and carotid arteries were harvested and snap-frozen in liquid nitrogen for RNA and protein extraction, or fixed with 4% PFA and processed for paraffin embedding for immunohistochemical analysis.

qPCR.

A cDNA fragment identified in differential mRNA display analysis of the rat carotid artery injury model (Sibinga et al., 1997) was cloned, sequenced, and subjected to BLAST analysis, which revealed homology of the sequence fragment with the 3' end of the rat Fat1 ORF (Genbank NM_031819). Total RNA was extracted from vascular tissues by homogenization in TRIzol (Invitrogen), treated with DNase 1 (1 U/μl, Promega), and used for first-strand cDNA synthesis. The mRNA levels were quantified in triplicate by qPCR in the Mx3000P Real-Time PCR System with the Brilliant SYBR Green qPCR kit (Stratagene). Rat Fat1 specific primers for qPCR were 5'-CCCCTTCCAACTCTCCCTCA-3' (forward) (SEQ ID NO:3) and 5'-CAGGCTCTCCCGGGCACTGT-3' (reverse) (SEQ ID NO:4). PCR cycling conditions included 10 min at 95° C. for 1 cycle followed by 45 cycles at 95° C. for 30 s, 60° C. for 30 s, and 72° C. for 60 s. Dissociation curve analysis confirmed that signals corresponded to unique amplicons. Expression levels were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in RNA levels for each sample, obtained from parallel assays and analyzed using the comparative $\Delta\Delta C_t$ method (Bustin, 2000).

Western Analysis.

Fat1-specific antisera were raised in rabbits. A cDNA fragment encoding mouse Fat1 aa 4434-4587 was generated by PCR and cloned in frame with GST in the pGEX-2T plasmid. The resultant fusion protein was expressed in bacteria, purified by GST-sepharose affinity chromatography (Pharmacia), and used as an immunogen in a standard rabbit injection protocol (Cocalico Labs). Fat1-specific antiserum was purified by affinity chromatography performed sequentially on a GST column and a GST-Fat1 column. Antiserum specificity was evaluated by Western analysis of GST-Fat1 fusion protein and whole cell lysates from RASMCs (1:5000 dilution). Other mouse antibodies used were anti-β-catenin (1:100, E-5, Santa Cruz), anti-cyclin D1 (1:100, DCS-6, NeoMarkers), anti-FLAG M2 (1:5000, Sigma), and anti-c-myc (1:250, 9E10).

For protein analyses, cells or vascular tissue samples were homogenized and extracted in RIPA buffer with or without protease inhibitors. Whole cell lysate (30 μg) was separated by electrophoresis through 3-8% Novex Tris-acetate or 4-12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to Immobilon-P membrane (Millipore). After blocking in TBST (Tris pH 8.0, NaCl 150 mmol/L, and 0.1% Tween-20) plus 4% (w/v) non-fat milk, blots were incubated overnight at 4° C. with primary antibodies. The blots were then incubated with HRP-conjugated secondary antibody and activity was visualized by enhanced chemiluminescence (ECL, Amersham). Equivalent protein loading was evaluated with anti-α-tubulin (1:500, NeoMarkers), anti-lamin A/C (1:100, N-18, Santa Cruz) or anti-actin (1:100, C-11, Santa Cruz) antibodies.

Immunohistochemistry.

Rat carotid arterial sections (5 μm) were incubated overnight with anti-Fat1 antiserum (1:2000), washed extensively, and incubated with a 1:500 dilution of secondary antibody (biotinylated goat anti-rabbit IgG, DAKO). Slides were incubated with avidin and biotinylated HRP, developed with a peroxidase substrate solution (DAKO), and counterstained with hematoxylin (Fisher). Specificity of staining was confirmed by omission of the primary antibody. PCNA staining was performed with anti-PCNA (1:100, PC 10, LabVision), alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:200), and visualization with BM Purple substrate (Roche). Images were obtained using an Eclipse E600 microscope, 40×/NA 0.75 Plan objective, and Coolpix 5400 camera (Nikon).

Cell Culture.

Primary culture RASMCs were prepared as described (Sibing a et al., 1997) and maintained in Dulbecco's MEM (DMEM, Invitrogen) containing 10% FBS (HyClone), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mmol/L HEPES (pH 7.4, Sigma). RASMCs were passaged every 3 to 5 days, and used between 4 and 8 passages from harvest. Primary culture MASMCs were harvested from the aortas of 12 week old male FVB mice by enzymatic dissociation, evaluated by immunocytochemical analysis by using α smooth muscle actin antibody (1:400, Clone 1A4, NeoMarkers) and maintained in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. MASMCs were passaged every 2 to 4 days, and used between 4 and 8 passages from harvest. The A7r5 embryonic RASMC, 3T3, and 293T cell lines (American Tissue Type Collection) were cultured in DMEM containing 10% FBS. ATII was obtained from Sigma, and bFGF and PDGF-BB from Collaborative Biomedical. In stimulation experiments, the cells were made quiescent by incubation in medium containing 0.4% horse serum for 72 h prior to addition of the FBS or growth factor. Control cultures received an equivalent amount of vehicle. Whole cellular protein was extracted at designed time points.

RNA Interference.

The mouse Fat1 short interfering RNA (siRNA) templates were comprised of 19 bp sense sequences derived from GenBank Accession AJ250768 (position 4881, 5'-GGACCGAAGTCACCAAGTA-3' [SEQ ID NO:5]; position 5126, 5'-GCGACGCATTTAACATTAA-3' [SEQ ID NO:6]; position 6432, 5'-GCATGACACTTTAAATAAA-3' [SEQ ID NO:7]; position 7296; 5'-GTCTGGCAATGATCATAAA-3') [SEQ ID NO:8] followed by a 9 bp loop sequence, a 19 bp antisense sequence, and a T7 promoter sequence. Control

siRNAs included scrambled (GTAACCATAAACAG-GCATT—SEQ ID NO:9) and mismatched (GTCTG ATAATGCGCATAAA—SEQ ID NO:10) derivatives of the 7296 sequence, and an unrelated siRNA based on the Renilla luciferase sequence. siRNA was transcribed in vitro using the T7-MEGAshortscript™ kit (Ambion), and transfected with X-tremeGENE Reagent (Roche) according to manufacturer recommendations. Fat1 knockdown efficiency was assessed by Western analysis.

cDNA Constructs.

The mouse $Fat1_{IC}$ cDNA was generated by RT-PCR with primers containing HindIII and XbaI sites (underlined) to facilitate cloning: forward 5'-AAGCTTCTCTGCCGGAAGATGATCAGTCGG-3' (SEQ ID NO:11) and reverse 5'-TCTAGACACTTCCGTATGCTGCTGGGA-3' (SEQ ID NO:12). The product was subcloned into the p3XFLAG-CMV-14 expression vector (Sigma). The IL2R expression construct (LaFlamme et al., 1994) was used to construct a chimeric cDNA encoding the IL2R extracellular and transmembrane domains and the $Fat1_{IC}$, with or without an in frame 3XFLAG tag (IL2R-$Fat1_{IC}$-3XFLAG and IL2R-$Fat1_{IC}$, respectively). The IL2R-E-$cadherin_{IC}$-3XFLAG construct was produced using a similar strategy. The truncated FLAG-tagged $Fat1_{IC}$ constructs, $Fat_{4189-4587}$ and $Fat1_{4201-4587}$, were generated by PCR from the IL2R-$Fat1_{IC}$-3XFLAG template using forward primers 5'-CCATGGGC-CTCTGCCGGAAGATGATCAGT-3' (SEQ ID NO:13) and 5'-CCATGGGCCAGGCTGAACCTGAAGACAAAC-3' (SEQ ID NO:14) and the CMV24 reverse primer; the resulting fragments were cloned into pcDNA3.1v5 (Invitrogen). The FLAG-tagged N-cadherin and Myc-tagged β-catenin constructs were gifts from R. Hazan and R. Kemler, respectively. All constructs were confirmed by sequencing.

Retrovirus Preparation and Transduction.

The retrovirus system used is based on the IRES-GFP-RV constructs developed by K. Murphy (Washington University, St. Louis) and Phoenix ecotropic packing cells provided by G. Nolan (Stanford University). The IL2R-$Fat1_{IC}$ cDNA was inserted upstream of the encephalomyocarditis virus internal ribosomal entry sequence (IRES) and green fluorescent protein (GFP) ORF in the GFP-RV vector. A7r5 cells, MASMCs, or RASMCs (5×10^5) were infected with virus-containing supernatant in the presence of polybrene (8 μg/mL). Control cells transduced with virus encoding GFP alone or IL2R and GFP were generated in parallel, and FACS analysis of retroviral transduced cell lines indicated similar levels of GFP expression.

Cell Migration Assays.

Cell migration was assessed by 1) scratch wounding of monolayers and 2) with Transwell 24-well cell culture inserts with 8-μm pores (Costar). For the former, MASMCs transfected with control or Fat1-specific siRNA were grown to confluence, and monolayers were denuded similarly using a 1000 μl pipette tip. Photomicrographs of the same fields were obtained sequentially at 24 and 30 h after injury using a Nikon TMS microscope, Plan 4×/NA 0.13 DL objective, and Coolpix 5400 camera, and cellular progress was quantitated by planimetry of the denuded area and converted to distance migrated using NIH Image 1.63 software. For Transwell assays, quiescent cells were harvested, counted, and added (5×10^4/well) to the insert. Culture medium containing 10% FBS as chemotactic agent was added to the lower chamber. After 4 h, non-migrating cells were removed from upper filter surfaces, and the filter was washed, fixed, and stained. Six randomly selected 200× fields were then photographed and cells that had migrated to the underside of the filter were counted.

Cell Proliferation Assays.

Cell number was evaluated with the CyQUANT Assay (Molecular Probes). Cells (2×10^4 per well) were plated in 6-well plates in DMEM containing 2% FBS, medium was replaced every other day, and at each time point, triplicate wells were washed with PBS and frozen at −80° C. Net sample fluorescence was determined on a Victor 2 plate reader (Wallac) and enumerated by reference to a standard curve. For the bromodeoxyuridine (BrdU) incorporation assay, cells plated on chamber slides (Becton-Dickinson) were serum-starved (0.4% horse serum) for 48 h and then stimulated with 10% FBS. BrdU (10 μM, Sigma) was added to cells for 6 h prior to harvest at 24 h. Cells were washed in PBS, fixed in 4% PFA, treated with HCl, and stained sequentially with anti-BrdU antibody (1:200, Abcam) and Alexa Fluor 555 conjugated secondary antibody (1:2000, Molecular Probes). Cells were counterstained with DAPI (Molecular Probes). Signals were visualized by fluorescence microscopy, and the numbers of BrdU-positive and total nuclei per field calculated.

Immunocytochemistry.

Cells were plated on chamber slides 24 h prior to staining, and then washed with PBS, fixed with PFA, blocked with 3% normal goat serum, and incubated with anti-β-catenin (1:100) and anti-Fat1 (1:1000) antibodies. Specific staining was identified with goat anti-mouse and chicken anti-rabbit IgG (Alexa Fluors, Molecular Probes). Expression of FLAG-tagged proteins was detected using FITC-conjugated anti-FLAG M2 antibody (8 μg/ml, Sigma). After counterstaining with DAPI, samples were mounted (Supermount medium, Biogenex) on glass slides and signals were visualized using an Olympus IX70 inverted fluorescent microscope equipped with 20×/NA 0.4 and 40×/NA 0.6 LWD objectives and standard fluorescent filter sets, a Cooke Sensicam CCD camera, and IPLab software (Scanalytics). Subsequent image processing was performed using Photoshop 7.0 and Illustrator 10.0 (Adobe Systems). Routine control experiments included omission of the primary antibodies. For Wnt pathway activation, cells were treated with LiCl (20 mmol/L) for 6-12 h, and then stained with anti-β-catenin antibody and DAPI nuclear stain.

Co-Immunoprecipitation.

Deletions within the $Fat1_{IC}$ portion of the IL2R-$Fat1_{IC}$-3XFLAG construct were engineered using the vector XbaI site and introducing NheI restriction sites (Quikchange mutagenesis, Stratagene) in frame at the following positions in the mouse Fat1 aa sequence: 4187, 4244, 4395, and 4497. The sequences between selected pairs of restriction sites were excised, plasmids recircularized, and constructs confirmed by sequencing. Plasmids were introduced into 293T cells using Lipofectamine 2000 (Invitrogen). Whole cell lysates were harvested 24 Ii after transfection in lysis buffer containing 50 mM Tris (pH7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, with protease inhibitors. Myc-tagged β-catenin was immunoprecipitated by incubating 400 μg of precleared lysate with 2 μg of c-Myc antibody for 2 h at 4° C., followed by incubation with protein G agarose (Invitrogen) at 4° C. overnight. For immunoprecipitation of endogenous proteins, RASMC whole cell lysates were precleared and then incubated with anti-Fat1 antiserum, anti-β-catenin antibody, or normal rabbit or mouse IgG for 2 h at 4° C., followed by incubation with protein G agarose overnight. The beads were washed and immune complexes recovered by boiling in sample buffer.

Fat1 and β-catenin were detected by Western analysis, as described above.

Cell Fractionation.

Membrane, cytoplasmic, and nuclear fractions were prepared using the Compartment Protein Extraction Kit (Chemicon) according to the manufacturer's instructions. Fractionation and loading of proteins was evaluated by western analysis with anti-lamin A/C antibody (Santa Cruz).

Analysis of Reporter Gene Activation.

A7r5 cells growing in DMEM supplemented with 10% FBS were transfected transiently using Lipofectamine 2000 with β-catenin, IL2R-Fat1$_{IC}$, Fat$_{4189-4587}$, Fat1$_{4201-4587}$ or control expression constructs, along with the TCF wild type (Topflash) and mutated control (Fopflash) luciferase reporter plasmids (Upstate Biotechnology), or cyclin D1 promoter luciferase reporter (Herber et al., 1994). MASMCs were transfected by Amaxa electroporation according to the manufacturer's instructions. The total amount of transfected DNA was kept constant. Cell lysates were harvested 24 h after transfection, and luciferase activity was determined using the Glo-lysis buffer system (Promega) and the Victor 2 plate reader. Luciferase activities were normalized to protein levels for each well. The data shown represent transfections repeated at least three times each.

Statistical Analysis.

Experiments were repeated at least three times. Data are presented as mean±SEM. Comparisons between 2 groups were analyzed by Student's t test, and comparisons between 3 or more groups were assessed by analysis of variance (ANOVA) with a Bonferroni/Dunn post hoc test. Significance was accepted for values of $P<0.05$.

Abbreviations List:

IC, intracellular; MASMC, mouse aortic smooth muscle cell; qPCR, quantitative PCR; RASMC, rat aortic smooth muscle cell; siRNA, small interfering RNA; VSMC, vascular smooth muscle cell.

SEQ ID NOs

SEQ ID NO: 1. Mouse Fat1 amino acid sequence - deduced from Refseq Genbank accession NM_001081286 (see also Genbank NP_001074755.1)

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   1 mqrhltllll lllflqqfgd sdgsqrlept ppiqfthfqy nvtvhensaa ktyvqhprkm
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 121 vkavekatda earakvrvqv ldtndlrplf sptsysvslp entairtsia rvsatdadig
 181 tngefyysfk drtdvfaihp tsgvvvltgr ldfletqlye leilaadrgm klygssgvss
 241 lakltvhveq anecapiita vtlspseldk dptyaiitve dcdqgangei aslsivagdl
 301 lqqfktvrsf pgskafkvka vgavdwdshp ygynltlqak dkqtppqfsp vkvvhiispq
 361 fragpvkfem dvyraeisef apphtpvvlv kaipsyshlr yvfksapgkp kfglnhntgl
 421 isilepirrq htshfelevt tsdkrasarv vvkvlgtnsn ppeftqtsyk asidenapig
 481 aavtrvsamd pdegengyvt ysianlnhvp fvidhftgtv stsenldyel mprvytlrir
 541 asdwglpyrr evevlatitl nnlndntplf erincegtip rdlgvgeqit tvsaidadel
 601 qlvryqieag neldlfglnp ssgvlslkhs ltdglgakvs fhslritatd genfatplyi
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1981 ftqdaysavv kensteartl avitaignpl neplfyriln pdrrfkisht sgvlsttgip

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2401 isehaahghf vmcvracdad ssdldkleys ilsgndhksf iidretgiit lsnlrrhtlk

2461 pfyslnvsvs dgvfrssarv nvtvmggnlh spvfhqneye velaenaplh tlvvqvkasd

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2761 rlkleksldh ettkwyqfsi larctlddye vvasidvsiq vkdandnspv lesspyeafi

2821 venlpggsrv iqirasdlds gangqvmysl dqsqdadiie sfainmetgw ittlkeldhe

2881 erasyqikvv asdhgekvql sstaivgvtv tdvndspprf taeiykgtvs eddppggvia

2941 ilsttdadte einrqvsyfi tggdalgqfa venvqsdwrv yvkkpldreq kdsylltvta

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3181 giiqlekhld relqavytlt lkavdqglpr rltatgtvvv svldindnpp vfeyreygas

3241 vsedivigte vlqvyaasrd ieanaeitya iisgnehgkf sidsktgaif iiesldyess

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3841 fvkyrllene nrlemklsmr lrtysshavv myargtdysi pgivsvqsiq vndgqwhavs

3901 levegnyakl vldevhtasg tapgalktln ldnyvffggh lrqqgtkhgr gaqvasgfrg

3961 cmdsiylngq elplnnkpra yahieewvdl shgclltate dcssspcqng gvcnpsptgg

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4261 dsrnnldrns fegsaipehp efstfnpesm hghrkavavc svapnlpppp psnspsdsds

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4381 aapdlskprg yhwdtsdwmp svplpdiqef pnyeaideht plysadpnai dtdyypggyd

4441 iesdfppppe dfpapdelpp lppefsdqfe sihpprdmpa agslgsssrs rqrfnlnqyl

4501 pnfypadmse pqkqgagens pcrepytpyp pgyqrnfeap tienmmnsvy astascsdvs

4561 accevesevm msdyesgddg hfeevtippl dsqqhtev

SEQ ID NO:2. Human Fat1 amino acid sequence - deduced from Refseq
Genbank accession NM_005245.3 (see also Genbank NP_005236.2)

1 mqrhlallll llllfqhfgd sdgsqrleqt plqfthleyn vtvqensaak tyvghpvkmg

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121 kalekntnve artkvrvqvl dtndlrplfs ptsysvslpe ntairtsiar vsatdadigt

181 ngefyysfkd rtdmfaihpt sgvivltgrl dyletklyem eilaadrgmk lygssgissm

241 akltvhieqa necapvitav tlspseldrd payaivtvdd cdqgangdia slsivagdll

301 qqfrtvrsfp gskeykvkai ggidwdshpf gynltlqakd kgtppqfssv kvihvtspqf

361 kagpvkfekd vyraeisefa ppntpvvmvk aipayshlry vfkstpgkak fslnyntgli

421 silepvkrqq aahfelevtt sdrkastkvl vkvlgansnp peftqtayka afdenvpigt

481 tvmslsavdp degengyvty sianlnhvpf aidhftgavs tsenldyelm prvytlrira

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601 lvqyqieagn eldffslnpn sgvlslkrsl mdglgakvsf hslritatdg enfatplyin

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721 ptgiqvkenq pvgssvifmn stdldtgfng klvyavsggn edscfmidme tgmlkilspl

781 drettdkytl nitvydlgip qkaawrllhv vvvdandnpp eflqesyfve vsedkevhse

841 iiqveatdkd lgpnghvtys ivtdtdtfsi dsvtgvvnia rpldrelqhe hslkieardq

901 areepqlfst vvvkvsledv ndnpptfipp nyrvkvredl pegtvimwle ahdpdlgqsg

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1141 epvyypeime nspkdvsvvq ieafdpdsss ndklmykits qnpqgffsih pktglittts

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1441 qvfikvidtn dhrpqfstsk yevvipedta peteilqisa vdqdeknkli ytlqssrdpl

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1921 gnigekfsmd yktgaltvqn ttqlrsryel tvrasdgrfa gltsvkinvk eskeshlkft

1981 qdvysavvke nsteaetlav itaignpine plfyhilnpd rrfkisrtsg vlsttgtpfd

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2341 lisllrtldy eqsrqhtifv ravdggmptl ssdvivtvdv tdlndnpplf eqqiyearis

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2761 kleksldhet tkwyqfsila rctqddhemv asvdvsiqvk dandnspvfe sspyeafive

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4561 msdyesgddg hfeevtippl dsqqhtev

SEQ ID NO:3. Rat Fat1-specific forward primer
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SEQ ID NO:4. Rat Fat1-specific reverse primer
CAGGCTCTCCCGGGCACTGT

SEQ ID NO:5. Mouse Fat1 siRNA template
GGACCGAAGTCACCAAGTA

SEQ ID NO:6. Mouse Fat1 siRNA template
GCGACGCATTTAACATTAA

SEQ ID NO:7. Mouse Fat1 siRNA template
GCATGACACTTTAAATAAA

SEQ ID NO:8. Mouse Fat1 siRNA template
GTCTGGCAATGATCATAAA

SEQ ID NO:9. Control siRNA
GTAACCATAAACAGGCATT

SEQ ID NO:10. Control siRNA
GTCTGATAATGCGCATAAA

SEQ ID NO:11. Mouse $Fat1_{IC}$ RT-PCR forward primer. Restriction site is underlined.
AAGCTTCTCTGCCGGAAGATGATCAGTCGG

SEQ ID NO:12. Mouse $Fat1_{IC}$ RT-PCR reverse primer. Restriction site is underlined.
TCTAGACACTTCCGTATGCTGCTGGGA

SEQ ID NO:13. $Fat_{4189-4587}$ PCR forward primer. Restriction site is underlined.
CCATGGGCCTCTGCCGGAAGATGATCAGT. Restriction site is underlined.

SEQ ID NO:14. $Fat1_{4201-4587}$ PCR forward primer
CCATGGGCCAGGCTGAACCTGAAGACAAAC. Restriction site is underlined.

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In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

SEQUENCE LISTING

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Glu Asn Leu Phe Lys Ala Glu Glu Tyr Val Leu Gly Asp Phe Cys Phe
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Glu Leu Met Pro Arg Val Tyr Thr Leu Arg Ile Arg Ala Ser Asp Trp
    530                 535                 540

Gly Leu Pro Tyr Arg Arg Glu Val Glu Val Leu Ala Thr Ile Thr Leu
545                 550                 555                 560

Asn Asn Leu Asn Asp Asn Thr Pro Leu Phe Glu Arg Ile Asn Cys Glu
                565                 570                 575

Gly Thr Ile Pro Arg Asp Leu Gly Val Gly Glu Gln Ile Thr Thr Val
            580                 585                 590

Ser Ala Ile Asp Ala Asp Glu Leu Gln Leu Val Arg Tyr Gln Ile Glu
        595                 600                 605

Ala Gly Asn Glu Leu Asp Leu Phe Gly Leu Asn Pro Ser Ser Gly Val
    610                 615                 620

Leu Ser Leu Lys His Ser Leu Thr Asp Gly Leu Gly Ala Lys Val Ser
625                 630                 635                 640

Phe His Ser Leu Arg Ile Thr Ala Thr Asp Gly Glu Asn Phe Ala Thr
                645                 650                 655

Pro Leu Tyr Ile Asn Leu Thr Val Ala Ala Ser Arg Lys Pro Val Asn
            660                 665                 670

Leu Gln Cys Glu Glu Thr Gly Val Ala Lys Met Leu Ala Glu Lys Leu
        675                 680                 685

Leu Gln Ala Asn Lys Leu His Ser Gln Gly Asp Val Glu Asp Ile Phe
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    690                 695                 700

Phe Asp Ser Tyr Ser Val Asn Thr His Thr Pro Gln Phe Gly Val Thr
705                 710                 715                 720

Leu Pro Thr Gly Ile Glu Val Lys Glu Asn Leu Pro Val Gly Ala Asn
                725                 730                 735

Ile Leu Phe Met Asn Ala Thr Asp Leu Asp Ser Gly Phe Asn Gly Lys
            740                 745                 750

Leu Val Tyr Ala Ile Ser Gly Gly Asn Asp Asp Ser Cys Phe Thr Ile
        755                 760                 765

Asp Met Glu Thr Gly Val Leu Lys Val Leu Ser Pro Leu Asp Arg Glu
    770                 775                 780

Val Met Asp Lys Tyr Thr Leu Asn Ile Thr Val Tyr Asp Leu Gly Ile
785                 790                 795                 800

Pro Gln Arg Ala Ala Trp Arg Leu Leu Asp Val Thr Val Leu Asp Ala
                805                 810                 815

Asn Asp Asn Ala Pro Glu Phe Leu Gln Glu Ser Tyr Phe Val Glu Val
            820                 825                 830

Ser Glu Asp Lys Glu Val Asn Ser Glu Ile Ile Gln Val Glu Ala Thr
        835                 840                 845

Asp Lys Asp Leu Gly Pro Ser Gly His Val Thr Tyr Ala Ile Leu Thr
    850                 855                 860

Asp Thr Glu Lys Phe Ser Ile Asp Ser Met Thr Gly Val Val Lys Ile
865                 870                 875                 880

Ile Gln Pro Leu Asp Arg Glu Val Gln Pro Val His Tyr Leu Lys Ile
                885                 890                 895

Glu Ala Arg Asp Gln Ala Thr Glu Glu Pro Arg Leu Phe Ser Thr Val
            900                 905                 910

Leu Leu Lys Val Ser Leu Asp Asp Val Asn Asp Asn Pro Pro Arg Phe
        915                 920                 925

Ile Pro Pro Asn Tyr Ser Val Lys Val Arg Glu Asp Leu Pro Glu Gly
    930                 935                 940

Thr Ile Ile Met Trp Leu Glu Ala Tyr Asp Pro Asp Val Gly Gln Ser
945                 950                 955                 960

Ser Gln Val Arg Tyr Ser Leu Leu Asp His Gly Glu Gly His Phe Asp
                965                 970                 975

Val Asp Lys Leu Ser Gly Ala Val Arg Ile Val Gln Gln Leu Asp Phe
            980                 985                 990

Glu Lys Lys Gln Leu Tyr Asn Leu  Thr Val Arg Ala Lys  Asp Lys Gly
        995                 1000                1005

Lys Pro  Val Ser Leu Ser Ser  Thr Cys Tyr Val Glu  Val Glu Val
    1010                1015                1020

Val Asp  Val Asn Glu Asn Leu  His Thr Pro Val Phe  Ser Ser Phe
    1025                1030                1035

Val Glu  Lys Gly Val Val Lys  Glu Asp Val Pro Thr  Gly Ser Ser
    1040                1045                1050

Val Met  Thr Val Ser Ala His  Asp Glu Asp Thr Gly  Arg Asp Gly
    1055                1060                1065

Glu Ile  Arg Tyr Ser Ile Arg  Asp Gly Ser Gly Ile  Gly Val Phe
    1070                1075                1080

Arg Ile  Asp Glu Glu Thr Gly  Val Ile Glu Thr Ser  Asp Arg Leu
    1085                1090                1095

Asp Arg  Glu Ser Thr Ser His  Tyr Trp Leu Thr Val  Tyr Ala Thr
    1100                1105                1110
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Asp Gln  Gly Val Val Pro Leu  Ser Ser Phe Ile Glu  Val Tyr Ile
    1115                 1120                 1125

Glu Val  Glu Asp Val Asn Asp  Asn Ala Pro Gln Thr  Ser Glu Pro
    1130                 1135                 1140

Val Tyr  Tyr Pro Glu Ile Met  Glu Asn Ser Pro Lys  Asp Val Ser
    1145                 1150                 1155

Val Val  Gln Ile Glu Ala Phe  Asp Pro Asp Ser Ser  Ser Asn Asp
    1160                 1165                 1170

Lys Leu  Thr Tyr Arg Ile Thr  Ser Gly Asn Pro Gln  Gly Phe Phe
    1175                 1180                 1185

Ser Ile  His Pro Lys Thr Gly  Leu Ile Thr Thr Thr  Ser Arg Lys
    1190                 1195                 1200

Leu Asp  Arg Glu Gln Gln Asp  Glu His Ile Leu Glu  Val Thr Val
    1205                 1210                 1215

Thr Asp  Asn Gly Val Pro Pro  Arg Ser Thr Ile Ala  Arg Val Ile
    1220                 1225                 1230

Val Lys  Ile Leu Asp Glu Asn  Asp Asn Arg Pro Gln  Phe Leu Gln
    1235                 1240                 1245

Lys Phe  Tyr Lys Ile Arg Leu  Pro Glu Arg Glu Lys  Ala Asp Gly
    1250                 1255                 1260

Asp Arg  Ser Ala Ser Lys Arg  Glu Pro Leu Tyr Arg  Val Ile Ala
    1265                 1270                 1275

Ala Asp  Lys Asp Glu Gly Pro  Asn Ala Glu Leu Ser  Tyr Ser Ile
    1280                 1285                 1290

Glu Glu  Gly Asn Glu His Gly  Arg Phe Ser Ile Glu  Pro Lys Thr
    1295                 1300                 1305

Gly Val  Val Ser Ser Lys Lys  Phe Ser Ala Ala Gly  Glu Tyr Asp
    1310                 1315                 1320

Ile Leu  Ser Ile Lys Ala Val  Asp Asn Gly Arg Pro  Gln Lys Ser
    1325                 1330                 1335

Ser Thr  Thr Arg Leu His Ile  Glu Trp Ile Ser Lys  Pro Lys Pro
    1340                 1345                 1350

Ser Ser  Glu Pro Ile Ser Phe  Glu Glu Ser Val Phe  Ser Phe Thr
    1355                 1360                 1365

Val Met  Glu Ser Asp Pro Val  Ala His Met Ile Gly  Val Ile Ser
    1370                 1375                 1380

Val Glu  Pro Pro Gly Met Pro  Leu Trp Phe Asp Ile  Ile Gly Gly
    1385                 1390                 1395

Asn Tyr  Asp Ser His Phe Asp  Val Asp Lys Gly Thr  Gly Thr Ile
    1400                 1405                 1410

Ile Val  Ala Lys Pro Leu Asp  Ala Glu Gln Lys Ser  Ser Tyr Asn
    1415                 1420                 1425

Leu Thr  Val Glu Ala Thr Asp  Gly Thr Thr Thr Ile  Leu Thr Gln
    1430                 1435                 1440

Val Leu  Ile Lys Val Ile Asp  Thr Asn Asp His Arg  Pro Gln Phe
    1445                 1450                 1455

Ser Thr  Ser Lys Tyr Glu Val  Ala Val Pro Glu Asp  Thr Glu Pro
    1460                 1465                 1470

Glu Val  Glu Ile Leu Gln Ile  Ser Ala Val Asp Arg  Asp Glu Lys
    1475                 1480                 1485

Asn Lys  Leu Ile Tyr Thr Leu  Gln Ser Ser Ile Asp  Pro Ala Ser
    1490                 1495                 1500

Leu Lys  Lys Phe Arg Leu Asp  Pro Ala Thr Gly Ala  Leu Tyr Thr
    1505                 1510                 1515
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Ala Glu  Lys Leu Asp His Glu  Ala Ile His Gln His  Val Leu Thr
    1520                 1525                 1530

Val Met  Val Arg Asp Gln Asp  Val Pro Val Lys Arg  Asn Phe Ala
    1535                 1540                 1545

Arg Ile  Val Val Asn Val Ser  Asp Lys Asn Asp His  Ala Pro Trp
    1550                 1555                 1560

Phe Thr  Ser Pro Ser Tyr Asp  Gly Arg Val Tyr Glu  Ser Ala Ala
    1565                 1570                 1575

Val Gly  Ser Val Val Leu Gln  Val Thr Ala Leu Asp  Lys Asp Lys
    1580                 1585                 1590

Gly Arg  Asn Ala Glu Val Leu  Tyr Ser Ile Glu Ser  Gly Asn Ile
    1595                 1600                 1605

Gly Asn  Ser Phe Thr Ile Asp  Pro Ile Leu Gly Ser  Ile Lys Thr
    1610                 1615                 1620

Ala Arg  Glu Leu Asp Arg Ser  His Gln Val Asp Tyr  Asp Leu Met
    1625                 1630                 1635

Val Lys  Ala Thr Asp Lys Gly  Asp Pro Pro Met Ser  Glu Met Thr
    1640                 1645                 1650

Ser Val  Arg Ile Ala Val Thr  Val Ala Asp Asn Ala  Ser Pro Lys
    1655                 1660                 1665

Phe Thr  Ser Lys Glu Tyr Ser  Ala Glu Ile Ser Glu  Ala Ile Arg
    1670                 1675                 1680

Ile Gly  Ser Phe Val Gly Met  Val Ser Ala His Ser  Gln Ser Ser
    1685                 1690                 1695

Val Met  Tyr Glu Ile Arg Asp  Gly Asn Met Gly Asp  Ala Phe Asn
    1700                 1705                 1710

Ile Asn  Pro His Ser Gly Ser  Ile Ile Thr Gln Arg  Ala Leu Asp
    1715                 1720                 1725

Phe Glu  Thr Leu Pro Met Tyr  Ser Leu Thr Val Gln  Gly Thr Asn
    1730                 1735                 1740

Met Ala  Gly Leu Ser Thr Asn  Thr Thr Val Val Val  His Val Arg
    1745                 1750                 1755

Asp Glu  Asn Asp Asn Pro Pro  Val Phe Thr Gln Ala  Glu Tyr Ser
    1760                 1765                 1770

Gly Phe  Ile Ser Glu Ser Ala  Ser Val Asn Ser Val  Val Leu Thr
    1775                 1780                 1785

Asp Arg  Asn Val Pro Leu Val  Ile Arg Ala Thr Asp  Ala Asp Arg
    1790                 1795                 1800

Glu Ser  Asn Ala Leu Leu Val  Tyr Gln Ile Val Glu  Pro Ser Val
    1805                 1810                 1815

His Asn  Tyr Phe Ala Ile Asp  Pro Thr Thr Gly Ala  Ile Arg Thr
    1820                 1825                 1830

Val Leu  Ser Leu Asp Tyr Glu  Glu Thr His Ala Phe  His Phe Thr
    1835                 1840                 1845

Val Gln  Val His Asp Met Gly  Thr Pro Arg Leu Phe  Ala Glu Tyr
    1850                 1855                 1860

Ala Ala  Asn Val Thr Val His  Val Ile Asp Ile Asn  Asp Cys Pro
    1865                 1870                 1875

Pro Val  Phe Ser Lys Ser Leu  Tyr Glu Val Ser Leu  Leu Leu Pro
    1880                 1885                 1890

Thr Tyr  Arg Gly Val Asn Val  Ile Thr Val Asn Ala  Thr Asp Ala
    1895                 1900                 1905

Asp Ser  Lys Ala Phe Ser Gln  Val Met Tyr Ser Ile  Thr Glu Gly
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    1910                1915                1920

Asn Ile  Gly Glu Lys Phe Ser  Met Asp His Lys Thr  Gly Thr Ile
    1925                1930                1935

Ala Ile  Gln Asn Thr Thr Gln  Leu Arg Ser Arg Tyr  Glu Leu Thr
    1940                1945                1950

Val Arg  Ala Ser Asp Gly Arg  Phe Thr Ser Met Ala  Ser Val Lys
    1955                1960                1965

Ile Asn  Val Lys Glu Ser Arg  Glu Ser Pro Leu Lys  Phe Thr Gln
    1970                1975                1980

Asp Ala  Tyr Ser Ala Val Val  Lys Glu Asn Ser Thr  Glu Ala Arg
    1985                1990                1995

Thr Leu  Ala Val Ile Thr Ala  Ile Gly Asn Pro Leu  Asn Glu Pro
    2000                2005                2010

Leu Phe  Tyr Arg Ile Leu Asn  Pro Asp Arg Arg Phe  Lys Ile Ser
    2015                2020                2025

His Thr  Ser Gly Val Leu Ser  Thr Thr Gly Ile Pro  Phe Asp Arg
    2030                2035                2040

Glu Gln  Gln Glu Thr Phe Asp  Val Val Val Glu Val  Thr Lys Glu
    2045                2050                2055

His Glu  Pro Ser Ala Val Ala  His Val Val Val Lys  Val Thr Val
    2060                2065                2070

Glu Asp  Gln Asn Asp Asn Ala  Pro Val Phe Val Asn  Leu Pro Tyr
    2075                2080                2085

Tyr Ala  Val Val Lys Val Asp  Ala Glu Val Gly His  Val Ile Arg
    2090                2095                2100

Tyr Val  Thr Ala Ile Asp Arg  Asp Ser Gly Arg Asn  Gly Asp Ile
    2105                2110                2115

His Tyr  Tyr Leu Lys Glu His  His Asp His Phe Gln  Ile Gly Pro
    2120                2125                2130

Ser Gly  Asp Ile Ser Leu Lys  Lys Gln Phe Glu His  Asp Thr Leu
    2135                2140                2145

Asn Lys  Glu Tyr Leu Val Thr  Val Val Ala Lys Asp  Gly Gly Ser
    2150                2155                2160

Pro Ala  Phe Ser Ala Glu Val  Leu Val Pro Ile Thr  Val Met Asn
    2165                2170                2175

Lys Ala  Met Pro Val Phe Glu  Lys Ala Phe Tyr Ser  Ala Glu Ile
    2180                2185                2190

Pro Glu  Asn Ile Gln Met His  Ser Pro Val Val His  Ile Gln Ala
    2195                2200                2205

Asn Ser  Pro Glu Gly Leu Lys  Val Phe Tyr Ser Ile  Thr Asp Gly
    2210                2215                2220

Asp Pro  Phe Ser Gln Phe Thr  Ile Asn Phe Asn Thr  Gly Val Val
    2225                2230                2235

Asn Val  Ile Ala Pro Leu Asp  Phe Glu Ser His Pro  Ala Tyr Lys
    2240                2245                2250

Leu Ser  Val Arg Ala Thr Asp  Ser Leu Thr Gly Ala  His Ala Glu
    2255                2260                2265

Val Phe  Val Asp Ile Ile Val  Glu Asp Ile Asn Asp  Asn Pro Pro
    2270                2275                2280

Val Phe  Val Gln Pro Ser Tyr  Ser Thr Thr Leu Ser  Glu Ala Ser
    2285                2290                2295

Val Ile  Gly Thr Pro Val Leu  Gln Val Arg Ala Thr  Asp Ser Asp
    2300                2305                2310
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Ser Glu  Pro Asn Arg Gly Ile  Ser Tyr Gln Leu Ile  Gly Asn His
    2315                 2320                 2325

Ser Lys  Ser His Asp His Phe  His Ile Asp Ser Asn  Thr Gly Leu
    2330                 2335                 2340

Ile Ser  Leu Val Arg Ala Leu  Asp Tyr Glu Gln Ser  Gln Gln His
    2345                 2350                 2355

Arg Ile  Phe Val Arg Ala Val  Asp Gly Gly Met Pro  Ala Leu Ser
    2360                 2365                 2370

Ser Asp  Val Val Val Thr Val  Ala Val Thr Asp Leu  Asn Asp Asn
    2375                 2380                 2385

Pro Pro  Leu Phe Glu Gln Gln  Val Tyr Glu Ala Arg  Ile Ser Glu
    2390                 2395                 2400

His Ala  Ala His Gly His Phe  Val Met Cys Val Arg  Ala Cys Asp
    2405                 2410                 2415

Ala Asp  Ser Ser Asp Leu Asp  Lys Leu Glu Tyr Ser  Ile Leu Ser
    2420                 2425                 2430

Gly Asn  Asp His Lys Ser Phe  Ile Ile Asp Arg Glu  Thr Gly Ile
    2435                 2440                 2445

Ile Thr  Leu Ser Asn Leu Arg  Arg His Thr Leu Lys  Pro Phe Tyr
    2450                 2455                 2460

Ser Leu  Asn Val Ser Val Ser  Asp Gly Val Phe Arg  Ser Ser Ala
    2465                 2470                 2475

Arg Val  Asn Val Thr Val Met  Gly Gly Asn Leu His  Ser Pro Val
    2480                 2485                 2490

Phe His  Gln Asn Glu Tyr Glu  Val Glu Leu Ala Glu  Asn Ala Pro
    2495                 2500                 2505

Leu His  Thr Leu Val Val Gln  Val Lys Ala Ser Asp  Arg Asp Ser
    2510                 2515                 2520

Gly Ile  Tyr Ser His Val Thr  Tyr His Ile Val Asn  Asp Phe Ala
    2525                 2530                 2535

Lys Asp  Arg Phe Tyr Val Asn  Asp Arg Gly Gln Ile  Phe Thr Leu
    2540                 2545                 2550

Glu Lys  Leu Asp Arg Glu Thr  Pro Ala Glu Lys Val  Ile Ser Ile
    2555                 2560                 2565

Arg Leu  Met Ala Lys Asp Ala  Gly Gly Lys Val Ala  Phe Cys Thr
    2570                 2575                 2580

Val Asn  Val Ile Leu Thr Asp  Asp Asn Asp Asn Ala  Pro Gln Phe
    2585                 2590                 2595

Arg Ser  Thr Lys Tyr Glu Val  Asn Ile Gly Ser Ser  Ala Ala Lys
    2600                 2605                 2610

Gly Thr  Ser Val Val Lys Val  Phe Ala Ser Asp Ala  Asp Glu Gly
    2615                 2620                 2625

Ser Asn  Ala Asp Val Thr Tyr  Ala Ile Glu Ala Asp  Ser Glu Ser
    2630                 2635                 2640

Val Lys  Glu Asn Leu Glu Ile  Asn Lys Leu Thr Gly  Leu Ile Thr
    2645                 2650                 2655

Thr Lys  Glu Ser Leu Ile Gly  Leu Glu Asn Glu Phe  Phe Thr Phe
    2660                 2665                 2670

Phe Val  Arg Ala Val Asp Ser  Gly Ser Pro Pro Arg  Glu Ser Val
    2675                 2680                 2685

Val Pro  Val Tyr Ile Lys Ile  Leu Pro Pro Glu Val  Gln Leu Pro
    2690                 2695                 2700

Arg Phe  Ser Glu Pro Phe Tyr  Thr Tyr Thr Ile Ser  Glu Asp Thr
    2705                 2710                 2715
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Pro Ile  Gly Thr Glu Ile Asp  Leu Ile Arg Val Glu  His Gly Gly
    2720                 2725                 2730

Ala Val  Leu Tyr Ile Leu Val  Lys Gly Asn Thr Pro  Glu Ser Asn
    2735                 2740                 2745

Arg Asp  Glu Phe Phe Val Ile  Asp Arg Gln Asn Gly  Arg Leu Lys
    2750                 2755                 2760

Leu Glu  Lys Ser Leu Asp His  Glu Thr Thr Lys Trp  Tyr Gln Phe
    2765                 2770                 2775

Ser Ile  Leu Ala Arg Cys Thr  Leu Asp Asp Tyr Glu  Val Val Ala
    2780                 2785                 2790

Ser Ile  Asp Val Ser Ile Gln  Val Lys Asp Ala Asn  Asp Asn Ser
    2795                 2800                 2805

Pro Val  Leu Glu Ser Ser Pro  Tyr Glu Ala Phe Ile  Val Glu Asn
    2810                 2815                 2820

Leu Pro  Gly Gly Ser Arg Val  Ile Gln Ile Arg Ala  Ser Asp Leu
    2825                 2830                 2835

Asp Ser  Gly Ala Asn Gly Gln  Val Met Tyr Ser Leu  Asp Gln Ser
    2840                 2845                 2850

Gln Asp  Ala Asp Ile Ile Glu  Ser Phe Ala Ile Asn  Met Glu Thr
    2855                 2860                 2865

Gly Trp  Ile Thr Thr Leu Lys  Glu Leu Asp His Glu  Glu Arg Ala
    2870                 2875                 2880

Ser Tyr  Gln Ile Lys Val Val  Ala Ser Asp His Gly  Glu Lys Val
    2885                 2890                 2895

Gln Leu  Ser Ser Thr Ala Ile  Val Gly Val Thr Val  Thr Asp Val
    2900                 2905                 2910

Asn Asp  Ser Pro Pro Arg Phe  Thr Ala Glu Ile Tyr  Lys Gly Thr
    2915                 2920                 2925

Val Ser  Glu Asp Asp Pro Pro  Gly Gly Val Ile Ala  Ile Leu Ser
    2930                 2935                 2940

Thr Thr  Asp Ala Asp Thr Glu  Glu Ile Asn Arg Gln  Val Ser Tyr
    2945                 2950                 2955

Phe Ile  Thr Gly Gly Asp Ala  Leu Gly Gln Phe Ala  Val Glu Asn
    2960                 2965                 2970

Val Gln  Ser Asp Trp Arg Val  Tyr Val Lys Lys Pro  Leu Asp Arg
    2975                 2980                 2985

Glu Gln  Lys Asp Ser Tyr Leu  Leu Thr Val Thr Ala  Thr Asp Gly
    2990                 2995                 3000

Thr Phe  Ser Ser Lys Ala Arg  Val Glu Val Lys Val  Leu Asp Ala
    3005                 3010                 3015

Asn Asp  Asn Ser Pro Val Cys  Glu Lys Thr Ser Tyr  Ser Asp Thr
    3020                 3025                 3030

Ile Pro  Glu Asp Ala Leu Pro  Gly Lys Leu Val Met  Gln Val Ser
    3035                 3040                 3045

Ala Thr  Asp Ala Asp Ile Arg  Ser Asn Ala Glu Ile  Thr Tyr Thr
    3050                 3055                 3060

Leu Phe  Gly Ser Gly Ala Glu  Lys Phe Lys Leu Asn  Pro Asp Thr
    3065                 3070                 3075

Gly Glu  Leu Arg Thr Leu Ala  Leu Leu Asp Arg Glu  Glu Gln Ala
    3080                 3085                 3090

Val Tyr  Asn Leu Leu Val Lys  Ala Thr Asp Gly Gly  Gly Arg Ser
    3095                 3100                 3105

Cys Gln  Ala Ala Ile Val Leu  Thr Leu Glu Asp Val  Asn Asp Asn
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    3110                3115                3120

Ala Pro  Glu Phe Thr Ala Glu  Pro Tyr Thr Ile Thr  Val Phe Glu
    3125                3130                3135

Asn Thr  Glu Pro Gly Thr Pro  Leu Thr Arg Val Gln  Ala Thr Asp
    3140                3145                3150

Ala Asp  Thr Gly Leu Asn Arg  Lys Ile Ser Tyr Ser  Leu Val Glu
    3155                3160                3165

Ser Ala  Asp Gly Gln Phe Ser  Ile Asn Glu Arg Ser  Gly Ile Ile
    3170                3175                3180

Gln Leu  Glu Lys His Leu Asp  Arg Glu Leu Gln Ala  Val Tyr Thr
    3185                3190                3195

Leu Thr  Leu Lys Ala Val Asp  Gln Gly Leu Pro Arg  Arg Leu Thr
    3200                3205                3210

Ala Thr  Gly Thr Val Val Val  Ser Val Leu Asp Ile  Asn Asp Asn
    3215                3220                3225

Pro Pro  Val Phe Glu Tyr Arg  Glu Tyr Gly Ala Ser  Val Ser Glu
    3230                3235                3240

Asp Ile  Val Ile Gly Thr Glu  Val Leu Gln Val Tyr  Ala Ala Ser
    3245                3250                3255

Arg Asp  Ile Glu Ala Asn Ala  Glu Ile Thr Tyr Ala  Ile Ile Ser
    3260                3265                3270

Gly Asn  Glu His Gly Lys Phe  Ser Ile Asp Ser Lys  Thr Gly Ala
    3275                3280                3285

Ile Phe  Ile Ile Glu Ser Leu  Asp Tyr Glu Ser Ser  His Glu Tyr
    3290                3295                3300

Tyr Leu  Thr Val Glu Ala Thr  Asp Gly Gly Thr Pro  Ser Leu Ser
    3305                3310                3315

Asp Val  Ala Thr Val Asn Ile  Asn Val Thr Asp Ile  Asn Asp Asn
    3320                3325                3330

Ser Pro  Val Phe Ser Gln Asp  Thr Tyr Thr Thr Val  Val Ser Glu
    3335                3340                3345

Asp Ala  Ala Leu Glu Gln Pro  Val Ile Thr Ile Met  Ala Asp Asp
    3350                3355                3360

Ala Asp  Gly Pro Ser Asn Ser  His Ile His Tyr Ser  Ile Ile Glu
    3365                3370                3375

Gly Asn  Gln Gly Ser Pro Phe  Thr Ile Asp Pro Val  Arg Gly Glu
    3380                3385                3390

Val Lys  Val Thr Lys Pro Leu  Asp Arg Glu Thr Ile  Ser Gly Tyr
    3395                3400                3405

Thr Leu  Thr Val Gln Ala Ala  Asp Asn Gly Asn Pro  Pro Arg Val
    3410                3415                3420

Asn Thr  Thr Thr Val Asn Ile  Asp Val Ser Asp Val  Asn Asp Asn
    3425                3430                3435

Ala Pro  Leu Phe Ser Arg Asp  Asn Tyr Ser Val Ile  Ile Gln Glu
    3440                3445                3450

Asn Lys  Pro Val Gly Phe Ser  Val Leu Lys Leu Val  Val Thr Asp
    3455                3460                3465

Lys Asp  Ser Ser His Asn Gly  Pro Pro Phe Phe Phe  Thr Ile Val
    3470                3475                3480

Ser Gly  Asn Asp Glu Asn Ala  Phe Glu Val Asn Gln  His Gly Val
    3485                3490                3495

Leu Leu  Thr Ala Ala Thr Ile  Lys Arg Lys Val Lys  Asp His Tyr
    3500                3505                3510
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Leu Leu  His Val Lys Val Ala  Asp Ser Gly Lys Pro  Gln Leu Ser
    3515                3520                3525

Ser Met  Thr His Ile Asp Ile  Arg Val Ile Glu Glu  Ser Ile His
    3530                3535                3540

Pro Pro  Ala Ile Leu Pro Leu  Glu Ile Phe Ile Thr  Ala Phe Gly
    3545                3550                3555

Glu Glu  Tyr Ser Gly Gly Val  Ile Gly Lys Ile His  Ala Thr Asp
    3560                3565                3570

Gln Asp  Val Tyr Asp Thr Leu  Met Tyr Ser Leu Asp  Pro His Met
    3575                3580                3585

Asp Gly  Leu Phe Ser Val Ser  Ser Thr Gly Gly Lys  Leu Ile Ala
    3590                3595                3600

His Arg  Lys Leu Asp Ile Gly  Gln Tyr Leu Leu Asn  Val Ser Val
    3605                3610                3615

Thr Asp  Gly Lys Phe Thr Thr  Val Ala Asp Ile Thr  Val His Ile
    3620                3625                3630

Gln Gln  Val Thr Gln Glu Met  Leu Asn His Thr Val  Ala Ile Arg
    3635                3640                3645

Phe Ala  Asn Leu Thr Pro Glu  Glu Phe Val Gly Asp  Tyr Trp Arg
    3650                3655                3660

Asn Phe  Gln Arg Ala Leu Arg  Asn Ile Leu Gly Val  Arg Lys Asn
    3665                3670                3675

Asp Ile  Gln Ile Val Ser Leu  Gln Pro Ser Glu Pro  His Ser His
    3680                3685                3690

Leu Asp  Val Leu Leu Phe Val  Glu Arg Ser Gly Gly  Thr His Val
    3695                3700                3705

Ser Thr  Lys Gln Leu Leu His  Lys Ile Asn Ser Ser  Val Thr Asp
    3710                3715                3720

Val Glu  Glu Ile Ile Gly Val  Arg Ile Leu Glu Val  Phe Gln Lys
    3725                3730                3735

Leu Cys  Ala Gly Leu Asp Cys  Pro Trp Lys Phe Cys  Asp Glu Lys
    3740                3745                3750

Val Ser  Val Asp Glu Asn Val  Met Ser Thr His Ser  Thr Ala Arg
    3755                3760                3765

Leu Ser  Phe Val Thr Pro Arg  His His Arg Thr Ala  Val Cys Leu
    3770                3775                3780

Cys Lys  Asp Gly Thr Cys Pro  Pro Val His His Gly  Cys Glu Asp
    3785                3790                3795

Asn Pro  Cys Pro Ala Gly Ser  Glu Cys Val Ala Asp  Pro Arg Glu
    3800                3805                3810

Glu Lys  Tyr Ser Cys Val Cys  Pro Gly Gly Gly Phe  Gly Lys Cys
    3815                3820                3825

Pro Gly  Ser Ser Ser Ile Thr  Phe Thr Gly Asn Ser  Phe Val Lys
    3830                3835                3840

Tyr Arg  Leu Leu Glu Asn Glu  Asn Arg Leu Glu Met  Lys Leu Ser
    3845                3850                3855

Met Arg  Leu Arg Thr Tyr Ser  Ser His Ala Val Val  Met Tyr Ala
    3860                3865                3870

Arg Gly  Thr Asp Tyr Ser Ile  Pro Gly Ile Val Ser  Val Gln Ser
    3875                3880                3885

Ile Gln  Val Asn Asp Gly Gln  Trp His Ala Val Ser  Leu Glu Val
    3890                3895                3900

Glu Gly  Asn Tyr Ala Lys Leu  Val Leu Asp Glu Val  His Thr Ala
    3905                3910                3915
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Ser Gly  Thr Ala Pro Gly Ala  Leu Lys Thr Leu Asn  Leu Asp Asn
    3920                 3925                 3930

Tyr Val  Phe Phe Gly Gly His  Leu Arg Gln Gln Gly  Thr Lys His
    3935                 3940                 3945

Gly Arg  Gly Ala Gln Val Ala  Ser Gly Phe Arg Gly  Cys Met Asp
    3950                 3955                 3960

Ser Ile  Tyr Leu Asn Gly Gln  Glu Leu Pro Leu Asn  Asn Lys Pro
    3965                 3970                 3975

Arg Ala  Tyr Ala His Ile Glu  Glu Trp Val Asp Leu  Ser His Gly
    3980                 3985                 3990

Cys Leu  Leu Thr Ala Thr Glu  Asp Cys Ser Ser Ser  Pro Cys Gln
    3995                 4000                 4005

Asn Gly  Gly Val Cys Asn Pro  Ser Pro Thr Gly Gly  Tyr Tyr Cys
    4010                 4015                 4020

Lys Cys  Ser Ala Leu Tyr Val  Gly Thr Phe Cys Glu  Val Ser Val
    4025                 4030                 4035

Asn Pro  Cys Ser Ser Asn Pro  Cys Leu Tyr Gly Gly  Thr Cys Met
    4040                 4045                 4050

Val Asp  Asn Gly Gly Phe Val  Cys Gln Cys Arg Gly  Leu Tyr Thr
    4055                 4060                 4065

Gly Leu  Val Leu Phe Phe Ser  Phe Cys Met Cys Phe  Ser Leu Phe
    4070                 4075                 4080

Arg Cys  Gln Leu Ser Pro Tyr  Cys Lys Asp Asp Pro  Cys Lys Asn
    4085                 4090                 4095

Gly Gly  Thr Cys Phe Asp Ser  Leu Asp Gly Ala Val  Cys Gln Cys
    4100                 4105                 4110

Asp Ser  Gly Phe Arg Gly Glu  Arg Cys Gln Ser Asp  Ile Asp Glu
    4115                 4120                 4125

Cys Ala  Gly Asn Pro Cys Arg  Asn Gly Ala Leu Cys  Glu Asn Thr
    4130                 4135                 4140

His Gly  Ser Tyr His Cys Asn  Cys Ser Gln Glu Tyr  Arg Gly Lys
    4145                 4150                 4155

His Cys  Glu Asp Ala Ser Pro  Asn His Tyr Val Ser  Thr Pro Trp
    4160                 4165                 4170

Asn Ile  Gly Leu Ala Glu Gly  Ile Gly Ile Ile Val  Phe Ile Ala
    4175                 4180                 4185

Gly Ile  Val Leu Leu Val Met  Val Phe Val Leu Cys  Arg Lys Met
    4190                 4195                 4200

Ile Ser  Arg Lys Lys Lys Arg  Gln Ala Glu Pro Glu  Asp Lys Arg
    4205                 4210                 4215

Leu Gly  Pro Thr Thr Ala Phe  Leu Gln Arg Pro Tyr  Phe Asp Ser
    4220                 4225                 4230

Lys Leu  Asn Lys Asn Ile Tyr  Ser Asp Ile Pro Pro  Gln Val Pro
    4235                 4240                 4245

Val Arg  Pro Ile Ser Tyr Thr  Pro Ser Ile Pro Ser  Asp Ser Arg
    4250                 4255                 4260

Asn Asn  Leu Asp Arg Asn Ser  Phe Glu Gly Ser Ala  Ile Pro Glu
    4265                 4270                 4275

His Pro  Glu Phe Ser Thr Phe  Asn Pro Glu Ser Met  His Gly His
    4280                 4285                 4290

Arg Lys  Ala Val Ala Val Cys  Ser Val Ala Pro Asn  Leu Pro Pro
    4295                 4300                 4305

Pro Pro  Pro Ser Asn Ser Pro  Ser Asp Ser Asp Ser  Ile Gln Lys
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    4310                4315                4320

Pro Ser  Trp Asp Phe Asp Tyr  Asp Ala Lys Val Val  Asp Leu Asp
    4325                4330                4335

Pro Cys  Leu Ser Lys Lys Pro  Leu Glu Glu Lys Pro  Ser Gln Pro
    4340                4345                4350

Tyr Ser  Ala Arg Glu Ser Leu  Ser Glu Val Gln Ser  Leu Ser Ser
    4355                4360                4365

Phe Gln  Ser Glu Ser Cys Asp  Asp Asn Glu Ser Leu  Ala Ala Pro
    4370                4375                4380

Asp Leu  Ser Lys Pro Arg Gly  Tyr His Trp Asp Thr  Ser Asp Trp
    4385                4390                4395

Met Pro  Ser Val Pro Leu Pro  Asp Ile Gln Glu Phe  Pro Asn Tyr
    4400                4405                4410

Glu Ala  Ile Asp Glu His Thr  Pro Leu Tyr Ser Ala  Asp Pro Asn
    4415                4420                4425

Ala Ile  Asp Thr Asp Tyr Tyr  Pro Gly Gly Tyr Asp  Ile Glu Ser
    4430                4435                4440

Asp Phe  Pro Pro Pro Pro Glu  Asp Phe Pro Ala Pro  Asp Glu Leu
    4445                4450                4455

Pro Pro  Leu Pro Pro Glu Phe  Ser Asp Gln Phe Glu  Ser Ile His
    4460                4465                4470

Pro Pro  Arg Asp Met Pro Ala  Ala Gly Ser Leu Gly  Ser Ser Ser
    4475                4480                4485

Arg Ser  Arg Gln Arg Phe Asn  Leu Asn Gln Tyr Leu  Pro Asn Phe
    4490                4495                4500

Tyr Pro  Ala Asp Met Ser Glu  Pro Gln Lys Gln Gly  Ala Gly Glu
    4505                4510                4515

Asn Ser  Pro Cys Arg Glu Pro  Tyr Thr Pro Tyr Pro  Pro Gly Tyr
    4520                4525                4530

Gln Arg  Asn Phe Glu Ala Pro  Thr Ile Glu Asn Met  Pro Met Ser
    4535                4540                4545

Val Tyr  Ala Ser Thr Ala Ser  Cys Ser Asp Val Ser  Ala Cys Cys
    4550                4555                4560

Glu Val  Glu Ser Glu Val Met  Met Ser Asp Tyr Glu  Ser Gly Asp
    4565                4570                4575

Asp Gly  His Phe Glu Glu Val  Thr Ile Pro Pro Leu  Asp Ser Gln
    4580                4585                4590

Gln His  Thr Glu Val
    4595


<210> SEQ ID NO 2
<211> LENGTH: 4588
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Met Gly Arg His Leu Ala Leu Leu Leu Leu Leu Leu Leu Leu Phe Gln
1               5                   10                  15

His Phe Gly Asp Ser Asp Gly Ser Gln Arg Leu Glu Gln Thr Pro Leu
            20                  25                  30

Gln Phe Thr His Leu Glu Tyr Asn Val Thr Val Gln Glu Asn Ser Ala
        35                  40                  45

Ala Lys Thr Tyr Val Gly His Pro Val Lys Met Gly Val Tyr Ile Thr
    50                  55                  60

His Pro Ala Trp Glu Val Arg Tyr Lys Ile Val Ser Gly Asp Ser Glu
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65                  70                  75                  80

Asn Leu Phe Lys Ala Glu Glu Tyr Ile Leu Gly Asp Phe Cys Phe Leu
                85                  90                  95

Arg Ile Arg Thr Lys Gly Gly Asn Thr Ala Ile Leu Asn Arg Glu Val
            100                 105                 110

Lys Asp His Tyr Thr Leu Ile Val Lys Ala Leu Glu Lys Asn Thr Asn
        115                 120                 125

Val Glu Ala Arg Thr Lys Val Arg Val Gln Val Leu Asp Thr Asn Asp
    130                 135                 140

Leu Arg Pro Leu Phe Ser Pro Thr Ser Tyr Ser Val Ser Leu Pro Glu
145                 150                 155                 160

Asn Thr Ala Ile Arg Thr Ser Ile Ala Arg Val Ser Ala Thr Asp Ala
                165                 170                 175

Asp Ile Gly Thr Asn Gly Glu Phe Tyr Tyr Ser Phe Lys Asp Arg Thr
            180                 185                 190

Asp Met Phe Ala Ile His Pro Thr Ser Gly Val Ile Val Leu Thr Gly
        195                 200                 205

Arg Leu Asp Tyr Leu Glu Thr Lys Leu Tyr Glu Met Glu Ile Leu Ala
    210                 215                 220

Ala Asp Arg Gly Met Lys Leu Tyr Gly Ser Ser Gly Ile Ser Ser Met
225                 230                 235                 240

Ala Lys Leu Thr Val His Ile Glu Gln Ala Asn Glu Cys Ala Pro Val
                245                 250                 255

Ile Thr Ala Val Thr Leu Ser Pro Ser Glu Leu Asp Arg Asp Pro Ala
            260                 265                 270

Tyr Ala Ile Val Thr Val Asp Asp Cys Asp Gln Gly Ala Asn Gly Asp
        275                 280                 285

Ile Ala Ser Leu Ser Ile Val Ala Gly Asp Leu Leu Gln Gln Phe Arg
    290                 295                 300

Thr Val Arg Ser Phe Pro Gly Ser Lys Glu Tyr Lys Val Lys Ala Ile
305                 310                 315                 320

Gly Gly Ile Asp Trp Asp Ser His Pro Phe Gly Tyr Asn Leu Thr Leu
                325                 330                 335

Gln Ala Lys Asp Lys Gly Thr Pro Pro Gln Phe Ser Ser Val Lys Val
            340                 345                 350

Ile His Val Thr Ser Pro Gln Phe Lys Ala Gly Pro Val Lys Phe Glu
        355                 360                 365

Lys Asp Val Tyr Arg Ala Glu Ile Ser Glu Phe Ala Pro Pro Asn Thr
    370                 375                 380

Pro Val Val Met Val Lys Ala Ile Pro Ala Tyr Ser His Leu Arg Tyr
385                 390                 395                 400

Val Phe Lys Ser Thr Pro Gly Lys Ala Lys Phe Ser Leu Asn Tyr Asn
                405                 410                 415

Thr Gly Leu Ile Ser Ile Leu Glu Pro Val Lys Arg Gln Gln Ala Ala
            420                 425                 430

His Phe Glu Leu Glu Val Thr Thr Ser Asp Arg Lys Ala Ser Thr Lys
        435                 440                 445

Val Leu Val Lys Val Leu Gly Ala Asn Ser Asn Pro Pro Glu Phe Thr
    450                 455                 460

Gln Thr Ala Tyr Lys Ala Ala Phe Asp Glu Asn Val Pro Ile Gly Thr
465                 470                 475                 480

Thr Val Met Ser Leu Ser Ala Val Asp Pro Asp Glu Gly Glu Asn Gly
                485                 490                 495
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Tyr Val Thr Tyr Ser Ile Ala Asn Leu Asn His Val Pro Phe Ala Ile
            500                 505                 510

Asp His Phe Thr Gly Ala Val Ser Thr Ser Glu Asn Leu Asp Tyr Glu
        515                 520                 525

Leu Met Pro Arg Val Tyr Thr Leu Arg Ile Arg Ala Ser Asp Trp Gly
    530                 535                 540

Leu Pro Tyr Arg Arg Glu Val Glu Val Leu Ala Thr Ile Thr Leu Asn
545                 550                 555                 560

Asn Leu Asn Asp Asn Thr Pro Leu Phe Glu Lys Ile Asn Cys Glu Gly
                565                 570                 575

Thr Ile Pro Arg Asp Leu Gly Val Gly Glu Gln Ile Thr Thr Val Ser
            580                 585                 590

Ala Ile Asp Ala Asp Glu Leu Gln Leu Val Gln Tyr Gln Ile Glu Ala
        595                 600                 605

Gly Asn Glu Leu Asp Phe Phe Ser Leu Asn Pro Asn Ser Gly Val Leu
    610                 615                 620

Ser Leu Lys Arg Ser Leu Met Asp Gly Leu Gly Ala Lys Val Ser Phe
625                 630                 635                 640

His Ser Leu Arg Ile Thr Ala Thr Asp Gly Glu Asn Phe Ala Thr Pro
                645                 650                 655

Leu Tyr Ile Asn Ile Thr Val Ala Ala Ser His Lys Leu Val Asn Leu
            660                 665                 670

Gln Cys Glu Glu Thr Gly Val Ala Lys Met Leu Ala Glu Lys Leu Leu
        675                 680                 685

Gln Ala Asn Lys Leu His Asn Gln Gly Glu Val Glu Asp Ile Phe Phe
    690                 695                 700

Asp Ser His Ser Val Asn Ala His Ile Pro Gln Phe Arg Ser Thr Leu
705                 710                 715                 720

Pro Thr Gly Ile Gln Val Lys Glu Asn Gln Pro Val Gly Ser Ser Val
                725                 730                 735

Ile Phe Met Asn Ser Thr Asp Leu Asp Thr Gly Phe Asn Gly Lys Leu
            740                 745                 750

Val Tyr Ala Val Ser Gly Gly Asn Glu Asp Ser Cys Phe Met Ile Asp
        755                 760                 765

Met Glu Thr Gly Met Leu Lys Ile Leu Ser Pro Leu Asp Arg Glu Thr
    770                 775                 780

Thr Asp Lys Tyr Thr Leu Asn Ile Thr Val Tyr Asp Leu Gly Ile Pro
785                 790                 795                 800

Gln Lys Ala Ala Trp Arg Leu Leu His Val Val Val Val Asp Ala Asn
                805                 810                 815

Asp Asn Pro Pro Glu Phe Leu Gln Glu Ser Tyr Phe Val Glu Val Ser
            820                 825                 830

Glu Asp Lys Glu Val His Ser Glu Ile Ile Gln Val Glu Ala Thr Asp
        835                 840                 845

Lys Asp Leu Gly Pro Asn Gly His Val Thr Tyr Ser Ile Val Thr Asp
    850                 855                 860

Thr Asp Thr Phe Ser Ile Asp Ser Val Thr Gly Val Val Asn Ile Ala
865                 870                 875                 880

Arg Pro Leu Asp Arg Glu Leu Gln His Glu His Ser Leu Lys Ile Glu
                885                 890                 895

Ala Arg Asp Gln Ala Arg Glu Glu Pro Gln Leu Phe Ser Thr Val Val
            900                 905                 910

Val Lys Val Ser Leu Glu Asp Val Asn Asp Asn Pro Pro Thr Phe Ile
        915                 920                 925
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Pro Pro Asn Tyr Arg Val Lys Val Arg Glu Asp Leu Pro Glu Gly Thr
    930                 935                 940

Val Ile Met Trp Leu Glu Ala His Asp Pro Asp Leu Gly Gln Ser Gly
945                 950                 955                 960

Gln Val Arg Tyr Ser Leu Leu Asp His Gly Glu Gly Asn Phe Asp Val
                965                 970                 975

Asp Lys Leu Ser Gly Ala Val Arg Ile Val Gln Gln Leu Asp Phe Glu
            980                 985                 990

Lys Lys Gln Val Tyr Asn Leu Thr  Val Arg Ala Lys Asp  Lys Gly Lys
        995                 1000                1005

Pro Val  Ser Leu Ser Ser Thr  Cys Tyr Val Glu Val  Glu Val Val
    1010                1015                1020

Asp Val  Asn Glu Asn Leu His  Pro Pro Val Phe Ser  Ser Phe Val
    1025                1030                1035

Glu Lys  Gly Thr Val Lys Glu  Asp Ala Pro Val Gly  Ser Leu Val
    1040                1045                1050

Met Thr  Val Ser Ala His Asp  Glu Asp Ala Arg Arg  Asp Gly Glu
    1055                1060                1065

Ile Arg  Tyr Ser Ile Arg Asp  Gly Ser Gly Val Gly  Val Phe Lys
    1070                1075                1080

Ile Gly  Glu Glu Thr Gly Val  Ile Glu Thr Ser Asp  Arg Leu Asp
    1085                1090                1095

Arg Glu  Ser Thr Ser His Tyr  Trp Leu Thr Val Phe  Ala Thr Asp
    1100                1105                1110

Gln Gly  Val Val Pro Leu Ser  Ser Phe Ile Glu Ile  Tyr Ile Glu
    1115                1120                1125

Val Glu  Asp Val Asn Asp Asn  Ala Pro Gln Thr Ser  Glu Pro Val
    1130                1135                1140

Tyr Tyr  Pro Glu Ile Met Glu  Asn Ser Pro Lys Asp  Val Ser Val
    1145                1150                1155

Val Gln  Ile Glu Ala Phe Asp  Pro Asp Ser Ser Ser  Asn Asp Lys
    1160                1165                1170

Leu Met  Tyr Lys Ile Thr Ser  Gly Asn Pro Gln Gly  Phe Phe Ser
    1175                1180                1185

Ile His  Pro Lys Thr Gly Leu  Ile Thr Thr Thr Ser  Arg Lys Leu
    1190                1195                1200

Asp Arg  Glu Gln Gln Asp Glu  His Ile Leu Glu Val  Thr Val Thr
    1205                1210                1215

Asp Asn  Gly Ser Pro Pro Lys  Ser Thr Ile Ala Arg  Val Ile Val
    1220                1225                1230

Lys Ile  Leu Asp Glu Asn Asp  Asn Lys Pro Gln Phe  Leu Gln Lys
    1235                1240                1245

Phe Tyr  Lys Ile Arg Leu Pro  Glu Arg Glu Lys Pro  Asp Arg Glu
    1250                1255                1260

Arg Asn  Ala Arg Arg Glu Pro  Leu Tyr His Val Ile  Ala Thr Asp
    1265                1270                1275

Lys Asp  Glu Gly Pro Asn Ala  Glu Ile Ser Tyr Ser  Ile Glu Asp
    1280                1285                1290

Gly Asn  Glu His Gly Lys Phe  Phe Ile Glu Pro Lys  Thr Gly Val
    1295                1300                1305

Val Ser  Ser Lys Arg Phe Ser  Ala Ala Gly Glu Tyr  Asp Ile Leu
    1310                1315                1320

Ser Ile  Lys Ala Val Asp Asn  Gly Arg Pro Gln Lys  Ser Ser Thr
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    1325                1330                1335

Thr Arg  Leu His Ile Glu Trp  Ile Ser Lys Pro Lys  Pro Ser Leu
    1340                1345                1350

Glu Pro  Ile Ser Phe Glu Glu  Ser Phe Phe Thr Phe  Thr Val Met
    1355                1360                1365

Glu Ser  Asp Pro Val Ala His  Met Ile Gly Val Ile  Ser Val Glu
    1370                1375                1380

Pro Pro  Gly Ile Pro Leu Trp  Phe Asp Ile Thr Gly  Gly Asn Tyr
    1385                1390                1395

Asp Ser  His Phe Asp Val Asp  Lys Gly Thr Gly Thr  Ile Ile Val
    1400                1405                1410

Ala Lys  Pro Leu Asp Ala Glu  Gln Lys Ser Asn Tyr  Asn Leu Thr
    1415                1420                1425

Val Glu  Ala Thr Asp Gly Thr  Thr Thr Ile Leu Thr  Gln Val Phe
    1430                1435                1440

Ile Lys  Val Ile Asp Thr Asn  Asp His Arg Pro Gln  Phe Ser Thr
    1445                1450                1455

Ser Lys  Tyr Glu Val Val Ile  Pro Glu Asp Thr Ala  Pro Glu Thr
    1460                1465                1470

Glu Ile  Leu Gln Ile Ser Ala  Val Asp Gln Asp Glu  Lys Asn Lys
    1475                1480                1485

Leu Ile  Tyr Thr Leu Gln Ser  Ser Arg Asp Pro Leu  Ser Leu Lys
    1490                1495                1500

Lys Phe  Arg Leu Asp Pro Ala  Thr Gly Ser Leu Tyr  Thr Ser Glu
    1505                1510                1515

Lys Leu  Asp His Glu Ala Val  His Gln His Thr Leu  Thr Val Met
    1520                1525                1530

Val Arg  Asp Gln Asp Val Pro  Val Lys Arg Asn Phe  Ala Arg Ile
    1535                1540                1545

Val Val  Asn Val Ser Asp Thr  Asn Asp His Ala Pro  Trp Phe Thr
    1550                1555                1560

Ala Ser  Ser Tyr Lys Gly Arg  Val Tyr Glu Ser Ala  Ala Val Gly
    1565                1570                1575

Ser Val  Val Leu Gln Val Thr  Ala Leu Asp Lys Asp  Lys Gly Lys
    1580                1585                1590

Asn Ala  Glu Val Leu Tyr Ser  Ile Glu Ser Gly Asn  Ile Gly Asn
    1595                1600                1605

Ser Phe  Met Ile Asp Pro Val  Leu Gly Ser Ile Lys  Thr Ala Lys
    1610                1615                1620

Glu Leu  Asp Arg Ser Asn Gln  Ala Glu Tyr Asp Leu  Met Val Lys
    1625                1630                1635

Ala Thr  Asp Lys Gly Ser Pro  Pro Met Ser Glu Ile  Thr Ser Val
    1640                1645                1650

Arg Ile  Phe Val Thr Ile Ala  Asp Asn Ala Ser Pro  Lys Phe Thr
    1655                1660                1665

Ser Lys  Glu Tyr Ser Val Glu  Leu Ser Glu Thr Val  Ser Ile Gly
    1670                1675                1680

Ser Phe  Val Gly Met Val Thr  Ala His Ser Gln Ser  Ser Val Val
    1685                1690                1695

Tyr Glu  Ile Lys Asp Gly Asn  Thr Gly Asp Ala Phe  Asp Ile Asn
    1700                1705                1710

Pro His  Ser Gly Thr Ile Ile  Thr Gln Lys Ala Leu  Asp Phe Glu
    1715                1720                1725
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Thr Leu  Pro Ile Tyr Thr Leu  Ile Ile Gln Gly Thr  Asn Met Ala
    1730                1735                1740

Gly Leu  Ser Thr Asn Thr Thr  Val Leu Val His Leu  Gln Asp Glu
    1745                1750                1755

Asn Asp  Asn Ala Pro Val Phe  Met Gln Ala Glu Tyr  Thr Gly Leu
    1760                1765                1770

Ile Ser  Glu Ser Ala Ser Ile  Asn Ser Val Val Leu  Thr Asp Arg
    1775                1780                1785

Asn Val  Pro Leu Val Ile Arg  Ala Ala Asp Ala Asp  Lys Asp Ser
    1790                1795                1800

Asn Ala  Leu Leu Val Tyr His  Ile Val Glu Pro Ser  Val His Thr
    1805                1810                1815

Tyr Phe  Ala Ile Asp Ser Ser  Thr Gly Ala Ile His  Thr Val Leu
    1820                1825                1830

Ser Leu  Asp Tyr Glu Glu Thr  Ser Ile Phe His Phe  Thr Val Gln
    1835                1840                1845

Val His  Asp Met Gly Thr Pro  Arg Leu Phe Ala Glu  Tyr Ala Ala
    1850                1855                1860

Asn Val  Thr Val His Val Ile  Asp Ile Asn Asp Cys  Pro Pro Val
    1865                1870                1875

Phe Ala  Lys Pro Leu Tyr Glu  Ala Ser Leu Leu Leu  Pro Thr Tyr
    1880                1885                1890

Lys Gly  Val Lys Val Ile Thr  Val Asn Ala Thr Asp  Ala Asp Ser
    1895                1900                1905

Ser Ala  Phe Ser Gln Leu Ile  Tyr Ser Ile Thr Glu  Gly Asn Ile
    1910                1915                1920

Gly Glu  Lys Phe Ser Met Asp  Tyr Lys Thr Gly Ala  Leu Thr Val
    1925                1930                1935

Gln Asn  Thr Thr Gln Leu Arg  Ser Arg Tyr Glu Leu  Thr Val Arg
    1940                1945                1950

Ala Ser  Asp Gly Arg Phe Ala  Gly Leu Thr Ser Val  Lys Ile Asn
    1955                1960                1965

Val Lys  Glu Ser Lys Glu Ser  His Leu Lys Phe Thr  Gln Asp Val
    1970                1975                1980

Tyr Ser  Ala Val Val Lys Glu  Asn Ser Thr Glu Ala  Glu Thr Leu
    1985                1990                1995

Ala Val  Ile Thr Ala Ile Gly  Asn Pro Ile Asn Glu  Pro Leu Phe
    2000                2005                2010

Tyr His  Ile Leu Asn Pro Asp  Arg Arg Phe Lys Ile  Ser Arg Thr
    2015                2020                2025

Ser Gly  Val Leu Ser Thr Thr  Gly Thr Pro Phe Asp  Arg Glu Gln
    2030                2035                2040

Gln Glu  Ala Phe Asp Val Val  Val Glu Val Thr Glu  Glu His Lys
    2045                2050                2055

Pro Ser  Ala Val Ala His Val  Val Val Lys Val Ile  Val Glu Asp
    2060                2065                2070

Gln Asn  Asp Asn Ala Pro Val  Phe Val Asn Leu Pro  Tyr Tyr Ala
    2075                2080                2085

Val Val  Lys Val Asp Thr Glu  Val Gly His Val Ile  Arg Tyr Val
    2090                2095                2100

Thr Ala  Val Asp Arg Asp Ser  Gly Arg Asn Gly Glu  Val His Tyr
    2105                2110                2115

Tyr Leu  Lys Glu His His Glu  His Phe Gln Ile Gly  Pro Leu Gly
    2120                2125                2130
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Glu Ile  Ser Leu Lys Lys Gln  Phe Glu Leu Asp Thr  Leu Asn Lys
    2135                 2140                 2145

Glu Tyr  Leu Val Thr Val Val  Ala Lys Asp Gly Gly  Asn Pro Ala
    2150                 2155                 2160

Phe Ser  Ala Glu Val Ile Val  Pro Ile Thr Val Met  Asn Lys Ala
    2165                 2170                 2175

Met Pro  Val Phe Glu Lys Pro  Phe Tyr Ser Ala Glu  Ile Ala Glu
    2180                 2185                 2190

Ser Ile  Gln Val His Ser Pro  Val Val His Val Gln  Ala Asn Ser
    2195                 2200                 2205

Pro Glu  Gly Leu Lys Val Phe  Tyr Ser Ile Thr Asp  Gly Asp Pro
    2210                 2215                 2220

Phe Ser  Gln Phe Thr Ile Asn  Phe Asn Thr Gly Val  Ile Asn Val
    2225                 2230                 2235

Ile Ala  Pro Leu Asp Phe Glu  Ala His Pro Ala Tyr  Lys Leu Ser
    2240                 2245                 2250

Ile Arg  Ala Thr Asp Ser Leu  Thr Gly Ala His Ala  Glu Val Phe
    2255                 2260                 2265

Val Asp  Ile Ile Val Asp Asp  Ile Asn Asp Asn Pro  Pro Val Phe
    2270                 2275                 2280

Ala Gln  Gln Ser Tyr Ala Val  Thr Leu Ser Glu Ala  Ser Val Ile
    2285                 2290                 2295

Gly Thr  Ser Val Val Gln Val  Arg Ala Thr Asp Ser  Asp Ser Glu
    2300                 2305                 2310

Pro Asn  Arg Gly Ile Ser Tyr  Gln Met Phe Gly Asn  His Ser Lys
    2315                 2320                 2325

Ser His  Asp His Phe His Val  Asp Ser Ser Thr Gly  Leu Ile Ser
    2330                 2335                 2340

Leu Leu  Arg Thr Leu Asp Tyr  Glu Gln Ser Arg Gln  His Thr Ile
    2345                 2350                 2355

Phe Val  Arg Ala Val Asp Gly  Gly Met Pro Thr Leu  Ser Ser Asp
    2360                 2365                 2370

Val Ile  Val Thr Val Asp Val  Thr Asp Leu Asn Asp  Asn Pro Pro
    2375                 2380                 2385

Leu Phe  Glu Gln Gln Ile Tyr  Glu Ala Arg Ile Ser  Glu His Ala
    2390                 2395                 2400

Pro His  Gly His Phe Val Thr  Cys Val Lys Ala Tyr  Asp Ala Asp
    2405                 2410                 2415

Ser Ser  Asp Ile Asp Lys Leu  Gln Tyr Ser Ile Leu  Ser Gly Asn
    2420                 2425                 2430

Asp His  Lys His Phe Val Ile  Asp Ser Ala Thr Gly  Ile Ile Thr
    2435                 2440                 2445

Leu Ser  Asn Leu His Arg His  Ala Leu Lys Pro Phe  Tyr Ser Leu
    2450                 2455                 2460

Asn Leu  Ser Val Ser Asp Gly  Val Phe Arg Ser Ser  Thr Gln Val
    2465                 2470                 2475

His Val  Thr Val Ile Gly Gly  Asn Leu His Ser Pro  Ala Phe Leu
    2480                 2485                 2490

Gln Asn  Glu Tyr Glu Val Glu  Leu Ala Glu Asn Ala  Pro Leu His
    2495                 2500                 2505

Thr Leu  Val Met Glu Val Lys  Thr Thr Asp Gly Asp  Ser Gly Ile
    2510                 2515                 2520

Tyr Gly  His Val Thr Tyr His  Ile Val Asn Asp Phe  Ala Lys Asp
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    2525                2530                2535

Arg Phe  Tyr Ile Asn Glu Arg  Gly Gln Ile Phe Thr  Leu Glu Lys
    2540                2545                2550

Leu Asp  Arg Glu Thr Pro Ala  Glu Lys Val Ile Ser  Val Arg Leu
    2555                2560                2565

Met Ala  Lys Asp Ala Gly Gly  Lys Val Ala Phe Cys  Thr Val Asn
    2570                2575                2580

Val Ile  Leu Thr Asp Asp Asn  Asp Asn Ala Pro Gln  Phe Arg Ala
    2585                2590                2595

Thr Lys  Tyr Glu Val Asn Ile  Gly Ser Ser Ala Ala  Lys Gly Thr
    2600                2605                2610

Ser Val  Val Lys Val Leu Ala  Ser Asp Ala Asp Glu  Gly Ser Asn
    2615                2620                2625

Ala Asp  Ile Thr Tyr Ala Ile  Glu Ala Asp Ser Glu  Ser Val Lys
    2630                2635                2640

Glu Asn  Leu Glu Ile Asn Lys  Leu Ser Gly Val Ile  Thr Thr Lys
    2645                2650                2655

Glu Ser  Leu Ile Gly Leu Glu  Asn Glu Phe Phe Thr  Phe Phe Val
    2660                2665                2670

Arg Ala  Val Asp Asn Gly Ser  Pro Ser Lys Glu Ser  Val Val Leu
    2675                2680                2685

Val Tyr  Val Lys Ile Leu Pro  Pro Glu Met Gln Leu  Pro Lys Phe
    2690                2695                2700

Ser Glu  Pro Phe Tyr Thr Phe  Thr Val Ser Glu Asp  Val Pro Ile
    2705                2710                2715

Gly Thr  Glu Ile Asp Leu Ile  Arg Ala Glu His Ser  Gly Thr Val
    2720                2725                2730

Leu Tyr  Ser Leu Val Lys Gly  Asn Thr Pro Glu Ser  Asn Arg Asp
    2735                2740                2745

Glu Ser  Phe Val Ile Asp Arg  Gln Ser Gly Arg Leu  Lys Leu Glu
    2750                2755                2760

Lys Ser  Leu Asp His Glu Thr  Thr Lys Trp Tyr Gln  Phe Ser Ile
    2765                2770                2775

Leu Ala  Arg Cys Thr Gln Asp  Asp His Glu Met Val  Ala Ser Val
    2780                2785                2790

Asp Val  Ser Ile Gln Val Lys  Asp Ala Asn Asp Asn  Ser Pro Val
    2795                2800                2805

Phe Glu  Ser Ser Pro Tyr Glu  Ala Phe Ile Val Glu  Asn Leu Pro
    2810                2815                2820

Gly Gly  Ser Arg Val Ile Gln  Ile Arg Ala Ser Asp  Ala Asp Ser
    2825                2830                2835

Gly Thr  Asn Gly Gln Val Met  Tyr Ser Leu Asp Gln  Ser Gln Ser
    2840                2845                2850

Val Glu  Val Ile Glu Ser Phe  Ala Ile Asn Met Glu  Thr Gly Trp
    2855                2860                2865

Ile Thr  Thr Leu Lys Glu Leu  Asp His Glu Lys Arg  Asp Asn Tyr
    2870                2875                2880

Gln Ile  Lys Val Val Ala Ser  Asp His Gly Glu Lys  Ile Gln Leu
    2885                2890                2895

Ser Ser  Thr Ala Ile Val Asp  Val Thr Val Thr Asp  Val Asn Asp
    2900                2905                2910

Ser Pro  Pro Arg Phe Thr Ala  Glu Ile Tyr Lys Gly  Thr Val Ser
    2915                2920                2925
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Glu Asp  Asp Pro Gln Gly Gly  Val Ile Ala Ile Leu  Ser Thr Thr
    2930                 2935                 2940

Asp Ala  Asp Ser Glu Glu Ile  Asn Arg Gln Val Thr  Tyr Phe Ile
    2945                 2950                 2955

Thr Gly  Gly Asp Pro Leu Gly  Gln Phe Ala Val Glu  Thr Ile Gln
    2960                 2965                 2970

Asn Glu  Trp Lys Val Tyr Val  Lys Lys Pro Leu Asp  Arg Glu Lys
    2975                 2980                 2985

Arg Asp  Asn Tyr Leu Leu Thr  Ile Thr Ala Thr Asp  Gly Thr Phe
    2990                 2995                 3000

Ser Ser  Lys Ala Ile Val Glu  Val Lys Val Leu Asp  Ala Asn Asp
    3005                 3010                 3015

Asn Ser  Pro Val Cys Glu Lys  Thr Leu Tyr Ser Asp  Thr Ile Pro
    3020                 3025                 3030

Glu Asp  Val Leu Pro Gly Lys  Leu Ile Met Gln Ile  Ser Ala Thr
    3035                 3040                 3045

Asp Ala  Asp Ile Arg Ser Asn  Ala Glu Ile Thr Tyr  Thr Leu Leu
    3050                 3055                 3060

Gly Ser  Gly Ala Glu Lys Phe  Lys Leu Asn Pro Asp  Thr Gly Glu
    3065                 3070                 3075

Leu Lys  Thr Ser Thr Pro Leu  Asp Arg Glu Glu Gln  Ala Val Tyr
    3080                 3085                 3090

His Leu  Leu Val Arg Ala Thr  Asp Gly Gly Gly Arg  Phe Cys Gln
    3095                 3100                 3105

Ala Ser  Ile Val Leu Thr Leu  Glu Asp Val Asn Asp  Asn Ala Pro
    3110                 3115                 3120

Glu Phe  Ser Ala Asp Pro Tyr  Ala Ile Thr Val Phe  Glu Asn Thr
    3125                 3130                 3135

Glu Pro  Gly Thr Leu Leu Thr  Arg Val Gln Ala Thr  Asp Ala Asp
    3140                 3145                 3150

Ala Gly  Leu Asn Arg Lys Ile  Leu Tyr Ser Leu Ile  Asp Ser Ala
    3155                 3160                 3165

Asp Gly  Gln Phe Ser Ile Asn  Glu Leu Ser Gly Ile  Ile Gln Leu
    3170                 3175                 3180

Glu Lys  Pro Leu Asp Arg Glu  Leu Gln Ala Val Tyr  Thr Leu Ser
    3185                 3190                 3195

Leu Lys  Ala Val Asp Gln Gly  Leu Pro Arg Arg Leu  Thr Ala Thr
    3200                 3205                 3210

Gly Thr  Val Ile Val Ser Val  Leu Asp Ile Asn Asp  Asn Pro Pro
    3215                 3220                 3225

Val Phe  Glu Tyr Arg Glu Tyr  Gly Ala Thr Val Ser  Glu Asp Ile
    3230                 3235                 3240

Leu Val  Gly Thr Glu Val Leu  Gln Val Tyr Ala Ala  Ser Arg Asp
    3245                 3250                 3255

Ile Glu  Ala Asn Ala Glu Ile  Thr Tyr Ser Ile Ile  Ser Gly Asn
    3260                 3265                 3270

Glu His  Gly Lys Phe Ser Ile  Asp Ser Lys Thr Gly  Ala Val Phe
    3275                 3280                 3285

Ile Ile  Glu Asn Leu Asp Tyr  Glu Ser Ser His Glu  Tyr Tyr Leu
    3290                 3295                 3300

Thr Val  Glu Ala Thr Asp Gly  Gly Thr Pro Ser Leu  Ser Asp Val
    3305                 3310                 3315

Ala Thr  Val Asn Val Asn Val  Thr Asp Ile Asn Asp  Asn Thr Pro
    3320                 3325                 3330
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Val Phe  Ser Gln Asp Thr Tyr  Thr Thr Val Ile Ser  Glu Asp Ala
    3335                 3340                 3345

Val Leu  Glu Gln Ser Val Ile  Thr Val Met Ala Asp  Asp Ala Asp
    3350                 3355                 3360

Gly Pro  Ser Asn Ser His Ile  His Tyr Ser Ile Ile  Asp Gly Asn
    3365                 3370                 3375

Gln Gly  Ser Ser Phe Thr Ile  Asp Pro Val Arg Gly  Glu Val Lys
    3380                 3385                 3390

Val Thr  Lys Leu Leu Asp Arg  Glu Thr Ile Ser Gly  Tyr Thr Leu
    3395                 3400                 3405

Thr Val  Gln Ala Ser Asp Asn  Gly Ser Pro Pro Arg  Val Asn Thr
    3410                 3415                 3420

Thr Thr  Val Asn Ile Asp Val  Ser Asp Val Asn Asp  Asn Ala Pro
    3425                 3430                 3435

Val Phe  Ser Arg Gly Asn Tyr  Ser Val Ile Ile Gln  Glu Asn Lys
    3440                 3445                 3450

Pro Val  Gly Phe Ser Val Leu  Gln Leu Val Val Thr  Asp Glu Asp
    3455                 3460                 3465

Ser Ser  His Asn Gly Pro Pro  Phe Phe Phe Thr Ile  Val Thr Gly
    3470                 3475                 3480

Asn Asp  Glu Lys Ala Phe Glu  Val Asn Pro Gln Gly  Val Leu Leu
    3485                 3490                 3495

Thr Ser  Ser Ala Ile Lys Arg  Lys Glu Lys Asp His  Tyr Leu Leu
    3500                 3505                 3510

Gln Val  Lys Val Ala Asp Asn  Gly Lys Pro Gln Leu  Ser Ser Leu
    3515                 3520                 3525

Thr Tyr  Ile Asp Ile Arg Val  Ile Glu Glu Ser Ile  Tyr Pro Pro
    3530                 3535                 3540

Ala Ile  Leu Pro Leu Glu Ile  Phe Ile Thr Ser Ser  Gly Glu Glu
    3545                 3550                 3555

Tyr Ser  Gly Gly Val Ile Gly  Lys Ile His Ala Thr  Asp Gln Asp
    3560                 3565                 3570

Val Tyr  Asp Thr Leu Thr Tyr  Ser Leu Asp Pro Gln  Met Asp Asn
    3575                 3580                 3585

Leu Phe  Ser Val Ser Ser Thr  Gly Gly Lys Leu Ile  Ala His Lys
    3590                 3595                 3600

Lys Leu  Asp Ile Gly Gln Tyr  Leu Leu Asn Val Ser  Val Thr Asp
    3605                 3610                 3615

Gly Lys  Phe Thr Thr Val Ala  Asp Ile Thr Val His  Ile Arg Gln
    3620                 3625                 3630

Val Thr  Gln Glu Met Leu Asn  His Thr Ile Ala Ile  Arg Phe Ala
    3635                 3640                 3645

Asn Leu  Thr Pro Glu Glu Phe  Val Gly Asp Tyr Trp  Arg Asn Phe
    3650                 3655                 3660

Gln Arg  Ala Leu Arg Asn Ile  Leu Gly Val Arg Arg  Asn Asp Ile
    3665                 3670                 3675

Gln Ile  Val Ser Leu Gln Ser  Ser Glu Pro His Pro  His Leu Asp
    3680                 3685                 3690

Val Leu  Leu Phe Val Glu Lys  Pro Gly Ser Ala Gln  Ile Ser Thr
    3695                 3700                 3705

Lys Gln  Leu Leu His Lys Ile  Asn Ser Ser Val Thr  Asp Ile Glu
    3710                 3715                 3720

Glu Ile  Ile Gly Val Arg Ile  Leu Asn Val Phe Gln  Lys Leu Cys
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    3725                3730                3735

Ala Gly  Leu Asp Cys Pro Trp  Lys Phe Cys Asp Glu  Lys Val Ser
    3740                3745                3750

Val Asp  Glu Ser Val Met Ser  Thr His Ser Thr Ala  Arg Leu Ser
    3755                3760                3765

Phe Val  Thr Pro Arg His His  Arg Ala Ala Val Cys  Leu Cys Lys
    3770                3775                3780

Glu Gly  Arg Cys Pro Pro Val  His His Gly Cys Glu  Asp Asp Pro
    3785                3790                3795

Cys Pro  Glu Gly Ser Glu Cys  Val Ser Asp Pro Trp  Glu Glu Lys
    3800                3805                3810

His Thr  Cys Val Cys Pro Ser  Gly Arg Phe Gly Gln  Cys Pro Gly
    3815                3820                3825

Ser Ser  Ser Met Thr Leu Thr  Gly Asn Ser Tyr Val  Lys Tyr Arg
    3830                3835                3840

Leu Thr  Glu Asn Glu Asn Lys  Leu Glu Met Lys Leu  Thr Met Arg
    3845                3850                3855

Leu Arg  Thr Tyr Ser Thr His  Ala Val Val Met Tyr  Ala Arg Gly
    3860                3865                3870

Thr Asp  Tyr Ser Ile Leu Glu  Ile His His Gly Arg  Leu Gln Tyr
    3875                3880                3885

Lys Phe  Asp Cys Gly Ser Gly  Pro Gly Ile Val Ser  Val Gln Ser
    3890                3895                3900

Ile Gln  Val Asn Asp Gly Gln  Trp His Ala Val Ala  Leu Glu Val
    3905                3910                3915

Asn Gly  Asn Tyr Ala Arg Leu  Val Leu Asp Gln Val  His Thr Ala
    3920                3925                3930

Ser Gly  Thr Ala Pro Gly Thr  Leu Lys Thr Leu Asn  Leu Asp Asn
    3935                3940                3945

Tyr Val  Phe Phe Gly Gly His  Ile Arg Gln Gln Gly  Thr Arg His
    3950                3955                3960

Gly Arg  Ser Pro Gln Val Gly  Asn Gly Phe Arg Gly  Cys Met Asp
    3965                3970                3975

Ser Ile  Tyr Leu Asn Gly Gln  Glu Leu Pro Leu Asn  Ser Lys Pro
    3980                3985                3990

Arg Ser  Tyr Ala His Ile Glu  Glu Ser Val Asp Val  Ser Pro Gly
    3995                4000                4005

Cys Phe  Leu Thr Ala Thr Glu  Asp Cys Ala Ser Asn  Pro Cys Gln
    4010                4015                4020

Asn Gly  Gly Val Cys Asn Pro  Ser Pro Ala Gly Gly  Tyr Tyr Cys
    4025                4030                4035

Lys Cys  Ser Ala Leu Tyr Ile  Gly Thr His Cys Glu  Ile Ser Val
    4040                4045                4050

Asn Pro  Cys Ser Ser Lys Pro  Cys Leu Tyr Gly Gly  Thr Cys Val
    4055                4060                4065

Val Asp  Asn Gly Gly Phe Val  Cys Gln Cys Arg Gly  Leu Tyr Thr
    4070                4075                4080

Gly Gln  Arg Cys Gln Leu Ser  Pro Tyr Cys Lys Asp  Glu Pro Cys
    4085                4090                4095

Lys Asn  Gly Gly Thr Cys Phe  Asp Ser Leu Asp Gly  Ala Val Cys
    4100                4105                4110

Gln Cys  Asp Ser Gly Phe Arg  Gly Glu Arg Cys Gln  Ser Asp Ile
    4115                4120                4125
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Asp Glu  Cys Ser Gly Asn Pro  Cys Leu His Gly Ala  Leu Cys Glu
    4130                 4135                 4140

Asn Thr  His Gly Ser Tyr His  Cys Asn Cys Ser His  Glu Tyr Arg
    4145                 4150                 4155

Gly Arg  His Cys Glu Asp Ala  Ala Pro Asn Gln Tyr  Val Ser Thr
    4160                 4165                 4170

Pro Trp  Asn Ile Gly Leu Ala  Glu Gly Ile Gly Ile  Val Val Phe
    4175                 4180                 4185

Val Ala  Gly Ile Phe Leu Leu  Val Val Val Phe Val  Leu Cys Arg
    4190                 4195                 4200

Lys Met  Ile Ser Arg Lys Lys  Lys His Gln Ala Glu  Pro Lys Asp
    4205                 4210                 4215

Lys His  Leu Gly Pro Ala Thr  Ala Phe Leu Gln Arg  Pro Tyr Phe
    4220                 4225                 4230

Asp Ser  Lys Leu Asn Lys Asn  Ile Tyr Ser Asp Ile  Pro Pro Gln
    4235                 4240                 4245

Val Pro  Val Arg Pro Ile Ser  Tyr Thr Pro Ser Ile  Pro Ser Asp
    4250                 4255                 4260

Ser Arg  Asn Asn Leu Asp Arg  Asn Ser Phe Glu Gly  Ser Ala Ile
    4265                 4270                 4275

Pro Glu  His Pro Glu Phe Ser  Thr Phe Asn Pro Glu  Ser Val His
    4280                 4285                 4290

Gly His  Arg Lys Ala Val Ala  Val Cys Ser Val Ala  Pro Asn Leu
    4295                 4300                 4305

Pro Pro  Pro Pro Pro Ser Asn  Ser Pro Ser Asp Ser  Asp Ser Ile
    4310                 4315                 4320

Gln Lys  Pro Ser Trp Asp Phe  Asp Tyr Asp Thr Lys  Val Val Asp
    4325                 4330                 4335

Leu Asp  Pro Cys Leu Ser Lys  Lys Pro Leu Glu Glu  Lys Pro Ser
    4340                 4345                 4350

Gln Pro  Tyr Ser Ala Arg Glu  Ser Leu Ser Glu Val  Gln Ser Leu
    4355                 4360                 4365

Ser Ser  Phe Gln Ser Glu Ser  Cys Asp Asp Asn Gly  Tyr His Trp
    4370                 4375                 4380

Asp Thr  Ser Asp Trp Met Pro  Ser Val Pro Leu Pro  Asp Ile Gln
    4385                 4390                 4395

Glu Phe  Pro Asn Tyr Glu Val  Ile Asp Glu Gln Thr  Pro Leu Tyr
    4400                 4405                 4410

Ser Ala  Asp Pro Asn Ala Ile  Asp Thr Asp Tyr Tyr  Pro Gly Gly
    4415                 4420                 4425

Tyr Asp  Ile Glu Ser Asp Phe  Pro Pro Pro Pro Glu  Asp Phe Pro
    4430                 4435                 4440

Ala Ala  Asp Glu Leu Pro Pro  Leu Pro Pro Glu Phe  Ser Asn Gln
    4445                 4450                 4455

Phe Glu  Ser Ile His Pro Pro  Arg Asp Met Pro Ala  Ala Gly Ser
    4460                 4465                 4470

Leu Gly  Ser Ser Ser Arg Asn  Arg Gln Arg Phe Asn  Leu Asn Gln
    4475                 4480                 4485

Tyr Leu  Pro Asn Phe Tyr Pro  Leu Asp Met Ser Glu  Pro Gln Thr
    4490                 4495                 4500

Lys Gly  Thr Gly Glu Asn Ser  Thr Cys Arg Glu Pro  His Ala Pro
    4505                 4510                 4515

Tyr Pro  Pro Gly Tyr Gln Arg  His Phe Glu Ala Pro  Ala Val Glu
    4520                 4525                 4530
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Ser Met Pro Met Ser Val Tyr Ala Ser Thr Ala Ser Cys Ser Asp
4535 4540 4545

Val Ser Ala Cys Cys Glu Val Glu Ser Glu Val Met Met Ser Asp
4550 4555 4560

Tyr Glu Ser Gly Asp Asp Gly His Phe Glu Glu Val Thr Ile Pro
4565 4570 4575

Pro Leu Asp Ser Gln Gln His Thr Glu Val
4580 4585

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What is claimed is:

1. A method of inhibiting vascular smooth muscle cell (VSMC) proliferation in a patient having a bypass surgery vascular repair or an angioplasty vascular repair, the method comprising administering to the patient having the vascular repair an amount of a fusion protein consisting essentially of the sequence of residues 4202-4588 of SEQ ID NO:2 fused to the amino acid sequence of an extracellular domain and transmembrane region of an interleukin 2 receptor α-chain which localizes to a cell membrane effective to inhibit (VSMC) proliferation.

2. The method of claim **1**, wherein the vascular repair is an angioplasty vascular repair.

3. The method of claim **1**, wherein the patient is at risk of restenosis.

4. The method of claim **1**, wherein the vascular repair is a bypass surgery vascular repair.

5. The method of claim **1**, wherein the vascular repair is of a coronary artery.

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